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BIOLOGICAL BULLETIN

PUBLISHED BY
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THE
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THE MARINE BIOLOGICAL LABORATORY

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I. TRUSTEES

Including Action of 1966 Annual Meeting

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Arnold Lazarow, University of Minnesota
Charles B. Metz, University of Miami
Keith R. Porter, Harvard University
George T. Scott, Oberlin College
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Howard A. Schneiderman, Western Reserve University

TO SERVE UNTIL 1967

Lester G. Barth, Marine Biological Laboratory
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Keith R. Porter
Teru Hayashi
Mac V. Edds, Jr.
Howard A. Schneiderman
Francis D. Carlson

II. ACT OF INCORPORATION

No. 3170

Commonwealth of Massachusetts

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, Henry B. Pierce, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[seal]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BYLAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

(Revised August 12, 1966)

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a Chairman of the Board of Trustees, President, Director, Treasurer and Clerk.

III. The Annual Meeting of the members shall be held on the Friday following the second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and nine Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these bylaws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Massachusetts. Special meetings of the Trustees shall be called by the Chairman, the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-six Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two consecutive terms of four years each, Trustees are ineligible for re-election until a year has elapsed.

(B) Trustees *ex officio,* who shall be the Chairman, the Director, the Treasurer, and the Clerk.

(C) Trustees *Emeriti,* who shall be elected from present or former Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeriti* shall have all the rights of the Trustees, except that *Trustees Emeriti* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation. They shall elect a Chairman of the Board of Trustees who shall be elected annually and shall serve until his successor is selected and qualified and who shall also preside at meetings of the Corporation. They shall elect a President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and Vice Chairman of meetings of the Corporation, and who shall be elected annually and shall serve until his successor is selected and qualified. They shall appoint a Director of the Laboratory for a term not to exceed five years, provided the term shall not exceed one year if the candidate has attained the age of 65 years prior to the date of the appointment. They may choose such other officers and agents as they may think best. They may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them except those chosen by the members, at any time. They may fill vacancies occurring in any manner in their own number or in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These bylaws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the bylaws will be acted upon.

RESOLUTIONS ADOPTED AT TRUSTEES' MEETING AUGUST 16, 1963—EXECUTIVE COMMITTEE

1. RESOLVED:

(A) The Executive Committee is hereby designated to consist of ten members as follows: *ex officio* members who shall be the Chairman of the Board of Trustees, President, Director and Treasurer; six additional Trustees, two of whom shall be elected by the Board of Trustees each year, to serve for a three-year term.

(B) The Chairman of the Board of Trustees shall act as Chairman of the Executive Commitee, and the President as Vice Chairman. (Adopted at the Trustees' Meeting, August 12, 1966.) A majority of the members of the Executive Committee shall constitute a quorum and a majority of those present at any properly held meeting shall determine its action. It shall meet at such times and places and upon such notice and appoint such sub-committees as the Committee shall determine.

(C) The Executive Committee shall have and may exercise all the powers of the Board during the intervals between meetings of the Board of Trustees except those powers specifically withheld from time to time by the Board or by Law.

(D) The Executive Committee shall keep appropriate minutes of its meetings, and its actions shall be reported to the Board of Trustees.

II. RESOLVED:

The elected members of the Executive Committee shall be constituted as a standing "Committee for the Nomination of Officers," responsible for making nominations at the Annual Meeting of the Corporation and of the Board of Trustees, for candidates to fill each office as the respective terms of office expire. (Chairman of the Board, President, Director, Treasurer, and Clerk.)

IV. REPORT OF THE DIRECTOR

To: The Trustees of the Marine Biological Laboratory

Gentlemen:

I submit herewith the report of the 79th session of the Marine Biological Laboratory. On this occasion I wish to pay tribute to the wise guidance of the administration of Dr. Armstrong from December, 1949, to mid-August, 1966. It is also my pleasure to note that Dr. Armstrong has consented to serve as Chairman of the Building Committee for the construction of the Training Building and the Dining-Dormitory complex.

1. *Facilities Development*

Late in December, 1966, we received notice that our facilities request, as outlined in the report for 1965, had been approved. The approval was in the form of a planning and design grant to allow us to proceed with final plans, drawings and specifications, up to a degree of completion that will allow asking for bids for construction. Upon the satisfactory completion of this first phase, the remainder of the sum requested will be made available.

Since we have no doubt of our ability to produce the proper base upon which to call for bids we may justifiably regard the past year as one which has seen the accomplishment of the funding of the final phases of our projected building program. These plans will be provided by the firm of Pierce, Pierce and Luyx, who are well known to us, under the supervision of Dr. Armstrong's Committee, Advisory to the Director and the Executive Committee.

2. *Winter Operation*

The use of our facilities throughout the year continues to grow. In recognition of numerous suggestions relating to the further services MBL might offer to biologists throughout the world, the Executive Committee authorized a Committee, composed of Dr. Edds, Chairman, Dr. Porter, Dr. Ebert, and the Director, *ex officio,* to explore the possibility of instituting special training programs and of finding an individual who might serve to give general direction to implementing the plans. Several meetings of this Committee have been held and the coming year will, in all probability, see specific cases both of individuals and of projects brought up for consideration.

This current winter there have been 27 investigators in residence at the Laboratory supplemented by a technical staff of 31. As usual, there have been many short-term visitors to use our Library, collect material and so forth. The Systematics-Ecology Program provides an active center in the appropriate areas, attracting visitors, holding seminars on the average of once a week and exchanging information with our neighbors in WHOI.

It seems quite certain, from the number of informal inquiries we receive, that the new dining hall-dormitory complex will be well used on a year-round basis when it becomes available.

3. *Systematics-Ecology Program*

This program was reviewed by our National Committee. Their advice has been most helpful. In general, progress has been good, especially as related to the biotic census and the museum collection. The Committee noted the cramped physical facilities for the museum and made strong representation for the necessity of inaugurating intellectually adventurous programs making full use of the fine physical facilities available for much of the year.

A highlight of the year was the delivery of the RV *Verrill*. This boat incorporates many novel design features and is greatly enhancing the SEP operations, especially relating to the biotic census.

4. *Organization*

As a result of the report of the Committee on Organization as discussed last season, Trustees have been canvassed for suggestions of names of individuals who might serve in the special group of Trustees. You will recall that members of this new group, one for each Class, were to be nominated by the Trustees and, while possessing the wisdom and interest in the MBL of the present Trustees, would not be subject to the usual residence requirements. Many suggestions have been screened by the Executive Committee and nominations were made by the Trustees at the winter meeting. Nominations will be presented for this special group at the Annual Meeting of 1967.

5. *MBL Policy*

The Marine Biological Laboratory at Woods Hole is a unique organization and facility. It exists to provide the best possible services for scientists who have programs of their own devising to carry out in our laboratories. Policy stems directly from the considerations of knowledgeable individuals, mostly practicing biological scientists who are members of the Corporation. Translation of policy into action is carried out through the Trustees, Executive Committee, Officers and Director.

In the present era of large programmatic operations all Corporation members and users of the Laboratory must be fully aware of past policy and future plans. As reported last year, the Corporation and Board of Trustees have reaffirmed past policies and noted that they apply without reference to the time of year or season, provided that long-term fixed programs which might interfere with the free flow of individual scientists be avoided.

6. *Instruction at the Marine Biological Laboratory*

Since its formation, instruction has been an integral part of the MBL program, inseparable from its devotion to fostering the well-being of scientific research. Instruction is carried out in many different ways, based, in the first instance, on the research laboratories of competent scientists who are training their own students. Over the years there also developed informal groups of individuals working in close proximity to each other and sharing equipment and ideas—for example, the several squid groups. The second stage toward organization of instruction is illustrated by the Training Grant programs—for example the Gamete and Fertilization program: and lastly, are the more formally announced and staffed courses. All levels of instruction are closely keyed to the progress of science, the courses by virtue of rotation of staff, each course director setting the orientation of the course, the other aspects being entirely dependent on the interest and dedication of individuals. This flexible system of instruction probably provides the most exciting atmosphere possible for young students of science. It is important to retain this flexibility.

7. *Personnel Changes*

The Laboratory was saddened at the opening of 1967 with the sudden death of Mr. Irvine L. Broadbent on January 9th, and Mr. Hallett Wagstaff on January 14th. Mr. Broadbent had served as Office Manager in the business office since 1954 and Mr. Wagstaff as the engine mechanic in the Supply Department since 1949.

Mr. Paul Shave, a collector in the Supply Department, moved to the Systematics-Ecology Program during the fall. Mr. Robert Hebden and Mr. Lewis Lawday have joined the Supply Department as collectors along with Mr. Edward Matthews as engine mechanics. In February Mr. Frank A. Wildes was appointed Office Manager.

8. *Gifts*

At the 1966 Annual Meeting, Mr. Daignault announced a gift in the amount of $50,000 from Mr. and Mrs. Swope in honor of Dr. Armstrong. This gift is unrestricted and may be used for any purpose felt by the Trustees to best honor the distinguished past services of Dr. Armstrong to the Laboratory.

In September of 1966, Mr. Perry E. Hall presented to the Laboratory his 42′ yacht *Tomahawk*. This gift is also without restrictions and the boat may be used or sold as will best serve the needs of the Laboratory.

1. Memorials

ETHEL BROWNE HARVEY

By E. G. Butler

With the death of Ethel Browne Harvey, the Marine Biological Laboratory suffered the loss of a gifted and devoted investigator, one whose close association with the Laboratory covered a period of nearly sixty years. She was born in Baltimore, Maryland, December 14, 1885, the daughter of a physician, Dr. Bennet B. Browne and his wife Jennie Nicholson Browne. She died in Falmouth, Massachusetts, September 2, 1965.

As a girl she received her early education at the Bryn Mawr School in Baltimore and then entered Goucher College from which she was graduated in 1906. In the autumn of that year she enrolled as a graduate student in zoology at Columbia University, receiving the M.A. degree in 1907, and the Ph.D. in 1913.

At Columbia she carried on advanced work primarily with E. B. Wilson and T. H. Morgan and soon developed firm research interests in the broad areas of cytology and embryology. While a graduate student she published six papers, one of them jointly with Professor Morgan. Her doctoral dissertation, carried out under the direction of Professor Wilson, was based on an extensive study of the cytology of the male germ cells of the hemipteran, *Notonecta*. Later papers were also concerned with the chromosome cytology of this organism and with problems of speciation.

Early in her career Ethel Harvey established an intimate association with the Marine Biological Laboratory. This was to continue throughout her life and here a major portion of her research was carried out. She first came to Woods Hole in the summer of 1906, directly after her graduation from Goucher, to take one of the courses being offered at that time. She returned as an investigator every summer during her graduate work at Columbia and in 1909, while still a graduate student, was elected to membership in the Corporation. Although in later years she carried on work at a number of other marine stations, notably the Stazione Zoologica in Naples, she regularly returned to Woods Hole for at least a portion of every summer; and usually she was here for the entire summer. In 1950, she became the first woman in a half century to be elected to the Board of Trustees; her only predecessor was Dr. Cornelia M. Clapp, elected in 1900. At the time of her death Dr. Harvey was a Trustee *Emeritus*.

Ethel Browne Harvey is undoubtedly best known and will long be remembered for her experimental work on the embryology of the sea urchin. Most of this research was carried out at Woods Hole. She turned to this subject in the early 1920's and her first paper, dealing with the effects of lack of oxygen on the development of *Arbacia,* was published in 1927. Soon thereafter she began to employ the techniques of centrifugation. After investigating the results of stratifying the cytoplasm of the sea urchin egg she turned her attention to the manner in which high centrifugation breaks an egg into separate fractions. Her studies on the developmental capacities of non-nucleate fractions led her to embark in the 1930's on an extensive study of the basic features of parthenogenetic merogony in the sea urchin; on this and associated phenomena she published a long series of papers.

To the preparation of her book, entitled "The American *Arbacia* and Other Sea Urchins," she devoted several years. Dedicated to the memory of her former teacher, Edmund B. Wilson, the book deals not alone with experimental work on sea urchin development, to which she was so devoted, but also to the distribution, behavior and general natural history of these organisms, as studied in ancient as well as in modern times. The erudition displayed in this book reveals Ethel Harvey at her scholarly best.

As a graduate student at Columbia and a summer research worker at Woods Hole, Ethel Browne became acquainted with E. Newton Harvey, who was working with Professor Morgan. They were married in 1916, after Newton Harvey had become a member of the Princeton University Faculty. Their honeymoon was a trip to Japan, where, characteristically, they spent a portion of the summer in research at the Misaki Marine Biological Station. Later, for many years they shared laboratories at Princeton and at Woods Hole. Two sons were born of the marriage, Edmund Newton Harvey, Jr., now a physical chemist, and Richard Bennet Harvey, a physician.

Over the years many honors came to Ethel Browne Harvey. She was elected to membership in the leading biological societies in this country and abroad. She was made a Fellow of the Institut International d'Embryologie and an Honorary Member of the

Societa Italiana de Biologia Sperimentale. In 1956 Goucher College conferred upon her the honorary degree of Doctor of Science.

It is proper to honor Ethel Browne Harvey in terms of her scientific capabilities and achievements. But to do so alone is to overlook other attributes which she possessed. A few persons will still recall her accomplishments as a swimmer; that as a young woman in Woods Hole she regularly took part in local swimming meets and, in particular, won the prizes for diving. She was also an accomplished tennis player. And, many will remember the corner table at the MBL Mess, presided over for so many years by Ethel and Newton Harvey, where animated conversation accompanied every meal. In a very real sense, Woods Hole was her scientific home; it was here that her most important work was accomplished; and, it was here that she enjoyed life in full measure.

JAMES H. WICKERSHAM

By C. Lloyd Claff

Born April 24, 1897, graduated from Yale University, 1918, with an A.B. degree. With the Equitable Trust Company, 1919–1921; Assistant Vice President Guaranty Trust Company, 1921–1934; Vice President and Head Investment Officer, Fifth Avenue Bank of New York, 1934–1965; President and Director, Fifth Avenue Bank Safe Deposit Vaults, Inc.; President and Director, Parish Safe Deposit Vaults, Inc., 791 Park Avenue; Treasurer and Trustee of the Marine Biological Laboratory, 1952–1965; Member of the Corporation of the Marine Biological Laboratory, 1952–1965; Chairman of the Board of Directors, General Biological Supply House, Chicago, Illinois; President of the Lennox Hill Hospital, 1952–1956; President, New York League for Hard of Hearing, 1950–1952. Served with the 31st Field Artillery, 1918–1919. Episcopalian; home, 791 Park Avenue, New York City, and Holiday House, Penzance Point, Woods Hole, Massachusetts.

Little do these vital statistics in "Who's Who in America" convey to the reader the real Jim Wickersham we were privileged to be associated with and know. He was Treasurer of the Marine Biological Laboratory for thirteen years, but very few of its members have any idea of the magnitude of his contribution to the welfare of the Corporation. When Jim became Treasurer, the Corporation had one million, one hundred and ten thousand dollars in an Irrevocable Trust Fund; and many small separate gift accounts amounting to $70,000. When he died in 1965, the Irrevocable Trust Fund had two and one-half million dollars and the pooled accounts of $70,000 had grown to $640,000. During this period there were generous gifts to the Laboratory so that all the growth was not due to Jim's effort, but it can be said without equivocation that his handling of the Fund added appreciably to it. One of the first things Jim did as Treasurer was to gather all the small gift funds into one pool, which by his adroit handling and constant supervision grew many times to its present generous proportions. He also made very helpful suggestions to the Trustees of the Irrevocable Fund.

Many tense moments in committee meetings were eased by some sagacious and timely remark of his. Jim Wickersham was not just a stern, skillful financier; his outgoing personality made him many friends. He was an ardent golfer, and avid bird watcher. He was Chairman of the Executive Committee of the Audubon Society. His interest in the Audubon Society took him to all parts of the United States, West Indies, the Bahamas, and Africa, constantly in search of more exotic birds.

He loved to travel and was a delightful traveling companion. One of these trips, with his wife Bertha, is recorded in Edward Streeter's book entitled "Along the Ridge," the joys and trials of two couples touring from Paris to Spain and on to Yugoslavia by automobile.

He seemed formidable at first but his associates soon learned he was kindly, and concerned himself with the comfort and peace of mind of those around him.

The untimely death of James H. Wickersham, shortly after retirement from a full and active business life, at a time when he could have given full rein to the journey through the various channels of his many and varied interests, challenges the mind of man, with the eternal mystery of life and justice.

ARTHUR KEMBLE PARPART

By Rudolf T. Kempton

The unexpected death of Arthur Kemble Parpart, President of the Marine Biological Laboratory, came with stunning suddenness on September 17, 1965, at the age of 61. Although a sense of loss is still acute, it is a source of deep satisfaction to realize the important role which he played in the operation and development of the Marine Biological Laboratory.

As an undergraduate at Amherst College he first attended the Laboratory in the summer of 1924 to take the Embryology Course. Later he took the Courses in Protozoology and Physiology, and worked at the Laboratory a total of forty-one consecutive summers. Perhaps it was the influence of Otto Glaser at Amherst, and that of Wallace Fenn, Leonor Michaelis, and especially Merkel Jacobs of the Physiology Course staff which directed Parpart into his lifetime interest in the physiology of the cell. After receiving the master's degree from Amherst he took his doctorate with Merkel Jacobs at the University of Pennsylvania, maintaining with him a research collaboration which extended over many years.

Upon completion of his work for the doctorate two significant events occurred: Dr. Parpart went to Princeton University as Instructor in Physiology, and he was elected to Membership in the Corporation of the Marine Biological Laboratory. These two institutions he was to serve loyally and effectively the rest of his life.

At the Marine Biological Laboratory he was called upon to serve on many *ad hoc* and standing committees. He was elected to the Board of Trustees in 1945, and except for such gaps as are required by the bylaws, served in this capacity thereafter. He was elected repeatedly by the Trustees to serve on the Executive Committee. In 1953 he became Vice President, and in 1963, he was made President of the Corporation.

Such a bald statement does little to suggest the devotion, the hard work, the constant thought which he placed in the service of the Laboratory. His interest in the welfare not only of the Corporation but of its personnel was deep and continuing. His interest was not restricted to the investigators, but included the members of the permanent staff, the students, the teaching faculty, the technical workers, the summer employees. He was concerned with the physical plant, educational programs, research facilities and the continued development of the Laboratory. He was influential in establishing the Lalor Program, and served on the first committee on the Lalor Fellows. He was interested in the Friday Evening Lectures, the Seminars and other general features of the Laboratory. The Library was a deep and continuing concern. It can be said truly that no phase of the Laboratory and its relationships was beyond his sphere of interest.

Paralleling his work at the Laboratory was his rise through the ranks at Princeton, and his service as Chairman of the Department of Biology from 1948 until his death. Still another phase included such diverse activities as Managing Editor of the Journal of Cellular and Comparative Physiology, the associate editorship of the American Scientist, chairmanship of a panel on cellular physiology for the National Research Council, membership in a study section of the National Institutes of Health, and service on the Advisory Board of the Brookhaven National Laboratory.

Undoubtedly less activity in these and other capacities would have left more time and energy for research. But this bibliography of valuable scientific contributions, listed in a Memorial in the Journal of Cellular and Comparative Physiology, and the graduate students whom he trained, are testimony that he managed to encompass a wide range of important activities.

In many ways he was a simple man, and certainly he was a modest one. Shortly after graduation from college he married his high school sweetheart, Ethel Roberta Bennett, a marriage which lasted until his death. His home, his wife and two children, books ranging from detective stories to serious general and scientific literature, together with occasional good music and quiet contact with friends, provided him with a satisfying environment. His love for sailing, gratified less frequently in his latter days, was acquired as a boy along the shores of Great South Bay on Long Island, and this love he passed on to his children.

He was essentially a gentle man. He could become irritated but he did not make others miserable by his irritation. He could be firm, especially when a principle was involved, but he could listen to and understand another point of view, perhaps while disagreeing with it completely. He could even accept as a legitimate point of view one which he simply could not understand.

There was laughter in his laboratory. It was not that the work was taken lightly; rather it was an interest in other persons, the ability to see the amusing side even of laboratory disasters, and perhaps it served as a release from all the varied pressures to which he was subjected.

An interesting and thoughtful view of the nature of this man is given by the following comments included in a letter written by one of his graduate students. "He was an extremely loyal and unusually kind person. With him the two went together. He was most accommodating to his students. . . . When I was at Princeton he was available seven days a week for conference, repairing of balky equipment or getting something set up. . . . I have seen him quite exasperated, but I do not recall in my years with him that he ever raised his voice at me. In brief, I think one could say that he was an outstanding teacher of graduate students and that by his own loyalty and kindness he brought out the best in his students . . . His scientific achievements represent a substantial contribution to erythrocyte physiology . . . His standards of scientific experimentation were of the highest quality and his ability to transmit them to students was of high order."

The Marine Biological Laboratory owes much to Arthur Kemble Parpart; he would have said that the debt was in the opposite direction.

SISTER FLORENCE MARIE SCOTT

By Donald P. Costello

Sister Florence Marie Scott, Professor of Biology at Seton Hill College and Trustee of the Marine Biological Laboratory, was born in Johnstown, Pennsylvania, on March 15, 1902, next to the youngest in a family of seven children. She was educated in the parochial schools of Pittsburgh, entered the Sisters of Charity in 1920, and received her Bachelor's degree from Seton Hill College in 1926, her Master's from Columbia University in 1927, and her Ph.D. from the same institution in 1935. Her major professor at Columbia was James H. McGregor and much of her early work on the development of tunicates was aided by E. G. Conklin. On the final examination for her doctoral degree, the committee consisted, among others, of E. B. Wilson, G. N. Calkins, Franz Schrader and L. G. Barth, all important figures in the history of the Marine Biological Laboratory. She was elected to the Corporation of this institution in 1942, and a Trustee in 1964, and she spent a total of 32 summers, or portions thereof, at Woods Hole, in addition to long stays during the autumn, winter and spring of several years. Her death, after a long illness, came on August 21, 1965.

This recital of facts can do nothing toward conveying the real essence of a woman who was warm and gay, sympathetic and thoughtful, courageous and steadfast in all things. Nor does it indicate the passionate loyalty she had for the Laboratory, its staff and its investigators, the deep concern she always felt for it in times of trouble and turmoil, her unwavering conviction that it has been, is, and will continue to be, a major factor in American and, indeed, in world biology. In a letter written less than a month before her death, one of her main concerns was about the forthcoming Corporation Meeting, and about all the homely little day-to-day happenings around the Laboratory. Another thing she especially asked about in this same letter was any impending birthdays, anniversaries, etc., among her friends here, and she expressd concern that she might not be able to remember such celebrants with notes. It was, I think, entirely fitting that at the Memorial Service held for her here in Woods Hole on August 26, the very large congregation included so many non-Catholics as to prompt the remark that she would have thought for a moment she was in the wrong church! This is an appropriate tribute to her as a member of her faith and her Congregation, and as a woman of very many deep and abiding friendships among all groups. It was also in the proper nature of things that the service should have been punctuated by the raucous screams of the gulls over Eel Pond, and by the whoop of the departing ferry—all noises familiar to her and cherished by her.

Sister Florence is sorely missed, but I think we are all the better for having known her. And the Marine Biological Laboratory remains very much in her debt, in ways both tangible and intangible.

WINTERTON C. CURTIS

By H. Burr Steinbach

With the death of Winterton C. Curtis on June 16, 1966, the Marine Biological Laboratory has lost the last of its 19th century Corporation members. Curtis first registered as a student in the Zoology Course at the Laboratory in 1896. In 1898 he was listed as an investigator here and in 1899 he was elected a member of the Corporation, a distinction he treasured for the rest of his life. He continued as an active research worker at the MBL for several decades, also serving as instructor, and later as head of the Zoology Course. He served on several of our committees and was elected a Trustee in 1923, becoming a Trustee *Emeritus* in 1946. He was a member of the Executive Committee from 1930 to 1932.

Dr. Curtis maintained a residence in Woods Hole, spending summers here during his active life even though, to his regret, other matters prevented his ever finishing his pioneer work on the effects of radiation on the regeneration of flatworms. He had a flair for recognizing things that should be done for the benefit of mankind through his understanding of the potentialities of science and he has left behind him a remarkable series of accomplishments and innovations. Perhaps the greatest scientific importance was his work through the National Research Council Committee on "The Effects of Radiation on Living Organisms," a Committee which he founded in 1928. In his capacity of Chairman of this Committee and, later, as Chairman of the Division of Biology and Agriculture of the National Research Council, he was responsible for obtaining equipment and funds to support the early work of such men as Muller, Stadler, Duggar and Failla. He also obtained the equipment and funds to install the x-ray laboratories here at the MBL, a service to biology which is now represented by the fourth floor of Whitman Laboratory, with the Failla cesium and cobalt sources and the radiation counting equipment. All this activity involved obtaining the permission of the National Research Council and the National Academy of Sciences, in 1928, to raise $175,000 for the committee sponsorship of research. This was a princely sum in those days.

Among other things, during his period of service to National Research Council, Curtis initiated and chaired a Conference of Biological Editors, an activity with a very modern title but unique for its time. A very wide audience knew W. C. Curtis as the senior author of the much used Curtis and Guthrie textbook of Zoology.

Curtis served as President of the American Society of Zoologists in 1932. He was also President of the old Union of Biological Sciences, an organization that labored mightily to foster the interests of all biology in the face of the splintering being brought about by the specialization of the researches of individual workers. During this time also, the Union was a moving force in the creation of Biological Abstracts.

With the true courage of the intellectual, he did not hesitate to climb down from the ivory tower and fight for what he thought was right and true. Thus he became one of the key expert witnesses for Darrow's defense of Scopes during the famous trial in Tennessee.

Shortly before his death, Dr. Curtis announced his intention of writing a candid history of the University of Missouri during the first quarter of a century. This would have been a memorable document since his whole academic life was concerned with the development of his home educational institution. From the time of his first appointment in 1901, he served the University as a faculty member, department chairman, Dean, and in other capacities as emergencies demanded. It was under his sponsorship that the University of Missouri showed pre-eminence at an early time in genetics and in cytogenetics.

W. C. Curtis should be well remembered by MBL folk. It was through his intervention that access to Stony Beach, *via* the Strong property, was preserved for Laboratory use. A timely option by him kept the lot from being sold for other uses and helped preserve the area for us to enjoy. Curtis was a great human being. We are the better for having had him with us.

2. The Staff

EMBRYOLOGY

I. Instructors

James D. Ebert, Director, Department of Embryology, Carnegie Institution of Washington, in charge of course
Allison L. Burnett, Associate Professor of Biology, Western Reserve University
James N. Cather, Associate Professor of Zoology, University of Michigan
Tom Humphreys, Assistant Professor of Biology, Massachusetts Institute of Technology
Thomas J. King, Head, Department of Embryology, Institute for Cancer Research, Philadelphia
Irwin R. Konigsberg, Staff Member, Department of Embryology, Carnegie Institution of Washington
James W. Lash, Associate Professor of Anatomy, University of Pennsylvania

II. Junior Instructor

Sidney B. Simpson, Department of Anatomy, Western Reserve University

III. Lecturers

Michael Abercrombie, Professor of Zoology, University College London, England
Donald D. Brown, Staff Member, Department of Embryology, Carnegie Institution of Washington
Clifford Grobstein, Professor of Biology, University of California, San Diego

IV. Laboratory Assistants

Burr G. Atkinson, Jr., University of Connecticut
William F. Sindelar, Western Reserve University

V. Lectures

Haverford College

J. D. Ebert	The keys to change: Factors regulating differentiation
E. Hadorn	The dynamics of determination and competence
C. H. Waddington	Fields and gradients
J. P. Trinkaus	Morphogenetic cell movements
H. Ursprung	Pattern formation
D. Koshland	Protein structure in relation to biological control mechanisms
A. Lang	Intercellular regulation in plants
J. W. Saunders, Jr.	Cell death in morphogenesis
H. Rubin	Oncogenesis
M. Jacobson	Ontogeny of behavior

Woods Hole

Thomas J. King	Analysis of early teleost development
	Analysis of developmental processes in teleosts
E. Anderson	Oocyte differentiation and vitellogenesis—a comparative study
R. A. Wallace	Vitellogenesis
L. Dennis Smith	Primordial germ cells
D. D. Brown	Biochemistry of oogenesis, fertilization and early development—I
	Biochemistry of oogenesis, fertilization and early development—II
L. Dennis Smith	Biochemistry of oogenesis, fertilization and early development—III
Tom Humphreys	Experimental analyses of selective cell association in marine sponges and vertebrate embryos
	Aspects of anatomy, physiology, reproduction and regeneration in sponges
	A direct analysis of species-specific cell association in marine sponges
A. L. Colwin	Early stages of fertilization: Sperm-egg relationships
M. Abercrombie	Contact-dependent behaviour in cell cultures on a solid substrate
Catherine Rappaport	A new approach to the design of *in vitro* conditions for differentiated mammalian cells
Leonard Weiss	The physicochemical bases of cell contact from a biological viewpoint
James Weston	The control of localization of migrating neural crest cells
A. Burnett	A model of cell differentiation in hydroids
	Pathways of cellular differentiation in hydroids
	Growth, polarity and form regulation in hydroids
J. J. Gilbert	Some aspects of form-change and sexuality in rotifers
G. Moment	Annelid regeneration: Problems and results

Hans Laufer	Analysis of interactions occurring during insect ontogeny
J. N. Cather	Annelids and Molluscs—I
	Annelids and Molluscs—II
	Annelids and Molluscs—III
J. Arnold	Development of squid
E. Bell	Biochemistry of early development in *Spisula*
J. W. Lash	Ascidians—I
	Ascidians—II
	Ascidians—III
Roger D. Milkman	Experimental studies on *Botryllus*
I. R. Konigsberg	Population effects in cell culture—I
	Population effects in cell culture—II
B. Goldberg	Collagen biosynthesis in cultured animal cells
G. Sato	Hormone biosynthesis in dispersed cell culture
C. Grobstein, J. W. Lash and A. J. Coulombre	MICROSYMPOSIUM on tissue interactions
S. B. Simpson, Jr.	Echinoderms—I
	Echinoderms—II
J. Papaconstantinou	Biochemistry of lens development
A. Monroy	Oogenesis and fertilization: Preliminaries to embryogenesis
G. Weissmann	Studies on lysosomes and artificial membranes
S. Gelfant	Studies on cell division
S. Cohen	Polyamines and control of nucleic acid synthesis
M. S. Kaulenas	RNA in the egg of *Ascaris lumbricoides*
David Kohne	The evolution of polynucleotide sequences
A. Novikoff	GERL in Neurons and Hepatocytes (Golgi associated region of endoplasmic reticulum from which lysosomes apparently form)
Y. Kato	Induced metaplasia in the extraembryonic membranes

PHYSIOLOGY

I. Consultants

Merkel H. Jacobs, Professor of Physiology, University of Pennsylvania

Albert Szent-Györgyi, Director, Institute for Muscle Research, Marine Biological Laboratory

W. D. McElroy, Director, McCollum-Pratt Institute, and Chairman, Department of Biology, The Johns Hopkins University

II. Instructors

J. Woodland Hastings, Professor of Biochemistry, University of Illinois, in charge of course

E. A. Adelberg, Professor of Microbiology, Yale University

Robert M. Bock, Professor of Biochemistry, University of Wisconsin

Herman Eisen, Professor of Microbiology, Washington University

Harlyn Halvorson, Professor of Bacteriology, University of Wisconsin, (On leave, 1966)

Shinya Inoué, Professor of Cytology, Dartmouth College

Fred Karush, Professor of Microbiology, University of Pennsylvania, (On leave, 1966)

Hans Kornberg, Professor of Biochemistry, Leicester University, England

K. E. Van Holde, Professor of Physical Chemistry, University of Illinois

III. Staff Associates

Carter Bancroft, Brandeis University
Ronald Cooper, Leicester University, England
Anatol Eberhard, Harvard University
Antonio Giuditta, International Laboratory of Genetics & Biophysics, Naples, Italy
Norman Klinman, University of Pennsylvania
J. Russell Little, Jr., Washington University Medical School
Emanuel J. Murgola, Yale University
Roland Rueckert, University of Wisconsin
Hidemi Sato, University of Pennsylvania
Raymond E. Stephens, Harvard University
James Wechsler, Yale University

IV. Special Lecturers

R. K. Clayton, C. F. Kettering Laboratory, Yellow Springs, Ohio
Quentin H. Gibson, Professor of Biochemistry, Cornell University, Ithaca, New York
Sol Spiegelman, Professor of Microbiology, University of Illinois
Alex Keynan, Israel Institute for Biological Research, Ness Ziona, Israel

V. Assistants

Carolyn Eberhard, Boston University
A. Randolph Sweeney, University of Illinois

VI. Lectures

J. W. Hastings	The generation and utilization of excited states in bioluminescent systems
	Enzymatic mechanisms in bioluminescence
Shinya Inoué	Dynamic structure and function of mitotic spindle—I
	Principles of polarized light microscepoy—II
	DNA arrangement in sperm nucleus—III
Donald Glaser	The control of events in the life cycle of *E. coli*
Robert Bock	The ribosome: Structure and function
	Codon recognition and translational regulation
Irwin Rose	Regulation of glycolysis in the red blood cell
K. E. Van Holde	The physical chemistry of macromolecules:
	I—Primary and secondary protein structure
	II—Tertiary protein structure
	III—Conformation of synthetic polynucleotides
Hans Kornberg	Integration of metabolism—I
	Integration of metabolism—II
	Integration of metabolism—III
Torsten Teorell	Some biophysical analysis on mechanoreceptors
Henry Mahler	Macromolecular synthesis and brain function: Report on Gordon Conference
Robert Bock	Molecular basis of learning and recall
A. Giuditta	Studies on rapidly-labeled RNA in rabbit brain
E. Kellenberger	Morphopoesis of the T-4 coli-phage
Q. H. Gibson	Hemoglobin-ligand reactions

E. A. Adelberg	Bacterial conjugation
	Genetics of suppression and regulation—I
	Genetics of suppression and regulation—II
Herman Eisen	Antibody-antigen interaction
	Antibody structure
	Antibody formation and other aspects of the immune response
Austin Riggs	Formation and properties of polymeric derivatives of some hemoglobins
Robert Forster	CO_2 reactions in the erythrocyte
B. D. Davis	Regulation of macromolecular biosynthesis
W. A. H. Rushton	Visual adaptation
David Shemin	Early steps in porphyrin synthesis: Mechanism and control
K. C. Atwood	Genetic mapping of templates for ribosomal RNA synthesis
Francis D. Carlson	Chemical energetics of muscular contraction
Cyrus Levinthal	Protein configuration—computer displays
Walter Vincent	Nucleolar involvement in ribosome synthesis
Gregorio Weber	Binding of small molecules to proteins
William Hagins	Fundamental excitatory processes in photoreceptors
Vincent Massey	Reaction mechanisms of flavo-protein enzymes
Howard K. Schachman	Recent developments and applications of the analytical ultracentrifuge
Rachmiel Levine	Mechanism of insulin action
Edward M. Kosower	Extrapolations from physical organic chemistry to biochemistry and biology: Some examples
Alex Keynan	The germination of the bacterial endospore: A case of unicellular differentiation
Paul Marks	Control of protein synthesis in developing erythroid cells
David Schlessinger	Some physiologic and genetic studies of *E. coli* ribosomes
R. K. Clayton	The bacterial photosynthetic reaction center
	Light emission and phosphorylation in photosynthesis

MARINE BOTANY

I. Consultant

William Randolph Taylor, Professor of Botany, University of Michigan

II. Instructors

Walter R. Herndon, Professor of Botany, University of Tennessee, in charge of course
Melvin S. Fuller, Associate Professor of Botany, University of California, Berkeley
H. Wayne Nichols, Associate Professor of Botany, Washington University
Janet Stein, Associate Professor of Botany, University of British Columbia, Vancouver

III. Special Lecturers

Philip Cook, University of Vermont
Paul Green, University of Pennsylvania
John Kingsbury, Cornell University
I. MacKenzie Lamb, Harvard University
Gordon Leedale, University of Leeds, England

Paul Levine, Harvard University
Luigi Provasoli, Haskins Laboratories
Frank Round, University of Bristol, England
Francis R. Trainor, University of Connecticut
Patricia L. Walne, University of Tennessee

IV. Assistants

Ailsie Baker, University of Tennessee
Davis L. Findley, University of Tennessee
Milton Sommerfeld, Washington University

V. Collector

Russell G. Rhodes, Kent University

VI. Lectures

Herndon	Systematics of marine plants
Fuller	Cyanophyta—I
Rhodes	Cyanophyta—II
Fuller	Cyanophyta—III (concluded)
	Introduction to fungi
Rhodes & Fuller	Isolation and cultivation of algae and fungi
Fuller	Marine bacteria
Rhodes	Chlorophyta: Tetrasporales
Herndon	Chlorophyta: Chlorococcales
Stein	Volvocales—I
	Volvocales—II
	Prasinophyceae
Fuller	Free-living marine phycomycetes
	Biology of marine phycomycetes
Herndon	Euglenophyta
Stein	Xanthophyceae—I
	Xanthophyceae—II
	Chrysophyceae
	Haptophyceae
Provasoli	Cultivation of marine algae
Nichols	Ulotrichales
Provasoli	Nutrition and external metabolites of marine algae
Nichols	Cladophorales
	Ulvales
Herndon	Siphonales
	Siphonocladales
	Dasycladales
Fuller	Fungi and marine algae
Herndon	Charophyta
Green	Growth and division of chloroplasts of *Nitella* (film)
Nichols	Rhodophyta—I
	Rhodophyta—II
	Development and ultrastructure
Fuller	Fungi and marine animals
Lamb	Antarctic benthic algae

Nichols	Rhodophyta—III
Taylor	Tropical algae
Herndon	Phaeophyta—I
	Phaeophyta—II
	Phaeophyta—III
Round	Bacillariophyceae
Fuller	Pyrrophyta
Levine	Algal mutants and photosynthesis
	Toward the resolution of the sequence of photosynthetic electron transport
Nichols	Rhodophyta—IV
	Rhodophyta—V
Herndon	Phaeophyta—IV
Nichols	Rhodophyta—VI
	Rhodophyta—VII (concluded)
Cook	Chlorophyta (concluded)
Kingsbury	Periodicity in *Derbesia halicystis*
Fuller	Marine ascomycetes
Round	Motility rhythms in benthic algae
Trainor	Pleomorphism in *Scenedesmus*
Leedale	*Euglena*
Walne	Eyespot of *Euglena* and *Chlamydomonas*

INVERTEBRATE ZOOLOGY

I. Consultants

Frank A. Brown, Jr., Morrison Professor of Biology, Northwestern University
Libbie H. Hyman, American Museum of Natural History
Clark P. Read, Professor of Biology, Rice University
Alfred C. Redfield, Woods Hole Oceanographic Institution

II. Instructors

W. D. Russell Hunter, Professor of Zoology, Syracuse University, in charge of course
George G. Holz, Jr., Professor of Microbiology, State University of New York, Upstate Medical Center
James D. Robertson, Titular Professor of Zoology, University of Glasgow, Scotland
Eric L. Mills, Assistant Professor of Biology, Queen's University, Kingston, Ontario, Canada
Frank M. Fisher, Jr., Assistant Professor of Biology, Rice University
Robert K. Josephson, Associate Professor of Biology, Western Reserve University
Jonathan P. Green, Assistant Professor, Division of Biological and Medical Sciences, Brown University
Meredith L. Jones, Curator in charge, Division of Worms, United States National Museum, Smithsonian Institute

III. Assistants

W. Bruce Hunter, University of California, Berkeley
Albert J. Burky, Syracuse University

IV. Lectures

Robert K. Josephson	Cnidaria I—Introduction to the Cnidaria and Ctenophora
	Cnidaria II—Feeding, growth, function of the nematocysts
W. D. Russell Hunter	Littoral ecology—Theoretical
George G. Holz, Jr.	Littoral ecology—Practical
Robert K. Josephson	Cnidaria III—Nervous system and behavior
	Seminar—Physiological mechanisms controlling behavior in the hydroid, *Tubularia*
Frank M. Fisher, Jr.	Turbellaria and Trematoda
	Cestoda and Rhynchocoela
Keith E. Dixon & James S. McDaniel	Physiological considerations of the host-parasite relationship
W. D. Russell Hunter	Mollusca I—General molluscan organization. Functioning of the mantle cavity in Gastropoda
	Mollusca II—Gastropoda (continued). Mantle cavity and feeding mechanisms in Bivalvia
	Seminar—Some problems of mechanics in molluscs
	Mollusca III—Adaptations in bivalves. Aspects of general physiology of gastropods and bivalves
W. D. Russell Hunter & Stephen C. Brown	Seminar—A partial history of the concept of segmentation in molluscs
W. D. Russell Hunter	Mollusca IV—Functional morphology in Amphineura, Cephalopoda and minor groups
Meredith L. Jones	Annelida introduction—General characteristics, classification, external morphology
W. D. Russell Hunter	Seminar—The evolution of, and physiological variation in the molluscs of fresh water
Meredith L. Jones	Annelida II—Feeding, respiration, osmoregulation
	Annelida III—Maturation, embryology
	Annelida IV—Nervous system, locomotion, endocrines
	Seminar—On the spatial distribution of selected benthic invertebrates, Pt. Richmond, California
Carl N. Schuster	Aspects of life-cycle and ecology of *Limulus*
Eric L. Mills	Arthropoda I—General features of arthropods. Introduction to crustacean structure
	Arthropoda II—Crustacean structure, physiology and reproduction
	Arthropoda III—Crustacean functional morphology and evolution
	Arthropoda IV—Crustacean functional morphology and evolution
H. Burr Steinbach (Chairman)	Symposium—Aspects of ionic regulation and excretion in invertebrate animals
Philip B. Dunham	The function of the contractile vacuole in *Tetrahymena*
W. T. W. Potts	Aspects of excretion in molluscs
James D. Robertson	Osmotic constituents of cephalopod blood and muscle
Frank M. Fisher, Jr.	Aschelminthes
Thomas J. M. Schopf	Biology of Ectoprocta
Arthur H. Clark	The composition and zoogeography of the deep-sea mollusk fauna

Frank M. Fisher, Jr.	Seminar—The gut and the parasite
James D. Robertson	Asteroidea—Anatomy and function; Ophiruoidea—Anatomy and function
	Echinoidea—Anatomy and function
	Seminar—Ionic regulation of body fluids in marine invertebrates
	Holothuroidea—Crinoidea—Development and larvae
Jonathan P. Green	Porifera
Ned Feder	Microtechnique for invertebrates: Histology and histochemistry
Jonathan P. Green	Protochordata I
James W. Lash & Roger D. Milkman	Urochordate development
Jonathan P. Green	Protochordata II
George G. Holz, Jr.	The nature of the Protozoa
	Flagellates I
	Flagellates II
	Sarcodines I
	Sarcodines II
Clyde J. Dawe	Neoplasia of invertebrate animals
George G. Holz, Jr.	Ciliates
Eric L. Mills	Seminar—The biology of an amphipod crustacean sibling species pair
W. D. Russell Hunter	One approach to the zooplankton

MARINE ECOLOGY

I. Consultants

Melbourne R. Carriker, Marine Biological Laboratory
Bostwick H. Ketchum, Woods Hole Oceanographic Institution
Edwin T. Moul, Rutgers University
John H. Ryther, Woods Hole Oceanographic Institution

II. Instructors

W. Rowland Taylor, Department of Oceanography and the Chesapeake Bay Institute, The Johns Hopkins University, in charge of course
J. R. Lewis, Department of Zoology, Leeds University, England
Howard L. Sanders, Woods Hole Oceanographic Institution
John Teal, Woods Hole Oceanographic Institution
Gotram Uhlig, Biologische Anstalt Helgoland, West Germany

III. Special Lecturer

Luigi Provasoli, Haskins Laboratories, New York

IV. Assistants

Claudia F. Bailey, Oberlin College
John F. Boyer, University of Chicago

V. Lectures

V. Zullo	Cape Cod as an ecological laboratory
W. Rowland Taylor	The Marine Environment—I Chemistry of sea water
	The Marine Environment—II Solar radiation through sea water
	Phytoplankton—I
	Phytoplankton—II
	Primary productivity by phytoplankton
	Phytoplankton—III
	Environment factors controlling primary productivity in the marine environment
	Light penetration through sediments and the physiology of intertidal diatoms
D. Grant	Specific diversity in the fauna of an intertidal sand community
D. Wall	Fossil and modern microplankton
J. A. Hellebust	Excretion and assimilation of organic substances by marine phytoplankton
E. R. Baylor	Plankton distribution and surface circulation
V. Zullo	Ecological adaptions in barnacles
Howard Sanders	Animal-sediment relationships
D. Rhodes	Animal effects on marine sediments
Charlotte Mangum	Evolutionary divergence in maldanid polychaetes
L. Provasoli	Special Lecture—Nutrition and external metabolites of marine algae
V. Zullo	Ecological adaption in barnacles
R. Scheltema	Larval dispersal of benthic invertebrates
L. Provasoli	Nutrient studies with axenic copepod cultures
Howard Sanders	Salinity, hydrography and the distribution of estuarine animals
	Biology of the deep sea benthos
	Factors determining diversity of animal assemblages
John Teal	Salt marsh ecology—I Origin and environmental conditions
	Salt marsh ecology—II Adaption of organisms to the marsh
	Salt marsh ecology—III Marsh productivity
	Some problems in ecology of pelagic marine life—gas exchange across sea surface
	Respiration of pelagic organisms
	Body temperature and activity in fish
G. Uhlig	Physiological ecology—a new field of research
	Effects of temperature on marine and brackish-water animals
	Effects of salinity and temperature on marine and brackish-water animals
	Methods of marine-biological research on benthonic protozoa
	The biology of *Noctiluca miliaris* (with movie)
	The biology of Folliculinidae (*Ciliata Heterotricha*) (with movie)

J. R. Lewis	Introduction to rocky shore ecology: The tidal environment Patterns of zonation Zonation: Modifying factors; causes Local and geographical distribution Problems in the distribution of *Chthamalus stellatus*

SYSTEMATICS-ECOLOGY PROGRAM

The Staff

Director: Melbourne R. Carriker
Resident Systematist & Assistant Director: Victor A. Zullo
Resident Ecologist: Robert H. Parker
Resident Biologist & Chief Diver: David C. Grant
Postdoctoral Fellows and Research Associates: Michael Ghiselin, Frank Round, Thomas J. M. Schopf, Joseph L. Simon, Barry A. Wade, David K. Young
Visiting Investigators in Residence: Louise Bush, Charlotte P. Mangum, Edwin T. Moul, Leif Størmer, Arnold Tamarin, Donald J. Zinn
Secretaries: Hazel F. Santos, Eva Monteiro
Artists: Diane Johnson, Linda Rogers, Ruth von Arx
Captain of Research Vessel: James P. Ostergard, Jr.
Student Participants: Keith Brander, Susan Burns, Betsy Palmer
Graduate Research Trainees: William R. Cobb, Charles D. Cox, Janice Czikowsky, Mae Teitelbaum
Research Assistants: Bruce Found, James M. Heller, Ross A. Kiester, Carol Kourtz, Penny Kuppinger, Franklyn Ott, Stuart L. Santos, Janis A. Speel, Dirk Van Zandt, Alan W. White
Biophotographer & Assistant Chief Diver: Peter J. Oldham
Curator, Gray Museum: Peter E. Schwamb

I. Seminars (winter included)

E. L. Bousfield	A biologist visits Russia and Poland
Barry A. Wade	Biology of the intertidal clam, *Donax*
Michael T. Ghiselin	The molluscan common ancestor in relation to methodological assumptions in comparative anatomy
Donald J. Zinn	History and biology of Penikese Island: 1872-present
Roland L. Wigley	Biological observations from a recent cruise of the *Albatross IV* to Georges Bank and the Gulf of Maine
Robert H. Parker	Applications of molluscan ecology to paleoecological problems
Alfred W. Senft	Studies in arginine metabolism of schistosomes
Peter J. Oldham	Biophotographic facilities of Systematics-Ecology Program
Peter Schwamb	The Marine Biological Laboratory-Systematics-Ecology Program Gray Museum: Functions and collections
David C. Grant	Cape Cod Bay: Plans and prospects
Jorgen Knudsen & Bent Hansen	Aspects of deep sea bivalves and holothurians
Clyde J. Dawe	Neoplasms of invertebrate animals

Victor Zullo	Origin and composition of the Galapagos Islands cirriped fauna
Arthur J. West	Development in the Phylum Acanthocephala
Bryant T. Sather	Studies on calcium and phosphorus metabolism in the crab, *Podophthalmus vigil*
Robert E. Johannes	External animal metabolites and the flux of energy and nutrients in marine ecosystems
Melbourne R. Carriker	Analysis of shell-boring behavior of *Urosalpinx cinera* (Say) by means of color motion pictures and micro hydrophone recording of radular sounds
Charlotte P. Mangum	Heme pigment synthesis in annelids—I
John Blake	What is happening at the Battelle Laboratories in Duxbury?
Charles F. Cole	Is there a marine program at the University of Massachusetts?
Donald J. Zinn	A beachcomber digs in (a very sifty lecture)
Thomas J. M. Schopf & Frank T. Manheim	Chemical composition of ectoprocts (Bryozoa)
William C. Summers	Some quantitative aspects of the feeding of fresh-water snails at the air-water surface
Arthur C. Borror	Marine ciliates of particulate and filamentous substrates
Robert P. Higgins	New lines in the kinorhynch industry
Roger H. Green	Regulation of an extremely dense bivalve population: The role of gastropod predation
Emery F. Swan	Some problems concerning growth and variation in echinoids
Nathan W. Riser	Systematics of nemerteans
Joseph L. Simon	Studies on the development of New England polychaetes, preliminary observations
Carl S. Hammen	Succinate dehydrogenase and fumarate reductase activity in bivalve molluscs and brachiopods
Victor A. Zullo	Evolutionary significance of deep sea barnacles
	Evolutionary divergence in maldanid polychaetes
Charlotte P. Mangum	Heme pigment synthesis in annelids—II
Randall B. Fairbanks	Ecological survey of the Cape Cod Canal in connection with the new hydro-electric power plant
M. Patricia Morse	Functional morphology of the digestive system in *Acanthodoris pilosa* (Mollusca: Opisthobranchia)
Victor A. Zullo	Darwin's contribution to cirripede systematics—I
Michael T. Ghiselin	Darwin's barnacles and systematic methodology—II
Mary P. Winsor	Barnacle larvae in the 19th century—III
Arnold Tamarin	Adaptation by two exocrine cell types to obstructed drainage in the rat submaxillary
R. O. Brinkhurst	The marine and brackish-water Tubificidae
Earl D. Hanson	Metazoan origins: Problems in phylogenetic inquiry
Leif Størmer	Geological history and evolution of horseshoe crabs and their kin
Robert R. Hessler	The origin of Crustacea
Eric L. Mills	Aristotle, Boeck, Sars and Stebbing: A history of systematic concepts in the amphipod crustaceans
Philip Person	Invertebrate cartilages

THE LABORATORY STAFF

Homer P. Smith, general manager

Irvine L. Broadbent, Office Manager
Miss Jane Fessenden, Librarian
Robert Kahler, Superintendent, Buildings and Grounds
Robert Gunning, Assistant Superintendent, Buildings and Grounds
Robert B. Mills, Manager, Department of Research Service
Carl O. Schweidenback, Manager, Supply Department
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Miss Katherine M. Tracy

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Bradley Wood

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Robert T. Martin, Manager, Food Service
Mrs. Elizabeth Kuil, Supervisor, Dining Room
Mrs. Ellen T. Nickelson, Supervisor, Dormitories
Alan G. Lunn, Supervisor, Cottage Colony

3. INVESTIGATORS: LALOR, LILLIE, GRASS AND RAND FELLOWS; STUDENTS

Independent Investigators, 1966

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Shepro, David, Professor of Biological Sciences, Simmons College and Boston University
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Steinbach, H. Burr, Chairman and Professor of Zoology, University of Chicago
Stephens, Raymond E., Postdoctoral Fellow, Dartmouth Medical School
Stephenson, William K., Chairman and Professor of Biology, Earlham College
Stillman, Irving M., Research Associate, National Institutes of Health
Størmer, Leif, Systematics-Ecology Program, Marine Biological Laboratory
Stretton, Anthony, O. W., Research Associate, Harvard Medical School
Strittmatter, Philipp, Associate Professor of Biochemistry, Washington University
Stunkard, Horace W., Research Associate, American Museum of Natural History
Sugi, Harvo, Research Associate, Columbia University
Surgenor, Douglas M., Dean, School of Medicine, State University of New York, at Buffalo
Szabó, George, Assistant Professor of Anatomy, Harvard Medical School
Szent-Györgyi, Albert, Director and Chief Investigator, The Institute for Muscle Research, Marine Biological Laboratory
Tamarin, Arnold, Systematics-Ecology Program, Marine Biological Laboratory
Tanzer, Marvin L., Research Associate, Massachusetts General Hospital and Harvard Medical School
Tasaki, Ichiji, Acting Chief, Laboratory of Neurobiology, National Institutes of Health
Taylor, Robert E., Acting Chief, Laboratory of Biophysics, National Institutes of Health
Taylor, W. Rowland, Assistant Professor of Oceanography, The Johns Hopkins University
Taylor, Wm. Randolph, Professor of Botany, University of Michigan
Torch, Reuben, Assistant Dean, College of Arts and Sciences, Professor of Biology, Oakland University
Trager, William, Professor, The Rockefeller University
Trinkaus, J. P., Director and Professor of Graduate Studies in Biology, Yale University
Troll, Walter, Associate Professor, New York University Medical Center
Uhlig, Rotram, Biologische Anstalt Helgoland, West Germany
Van Holde, K. E., Professor of Chemistry, University of Illinois
Venosa, Roque Alberto, Research Associate, Columbia University
Villee, Claude A., Andelot Professor of Biological Chemistry, Harvard University Medical School
Vincent, W. S., Associate Professor of Anatomy and Cell Biology, University of Pittsburgh
Wade, Barry A., Systematics-Ecology Program, Marine Biological Laboratory
Wald, George, Professor of Biology, Harvard University
Wallace, Robin A., Staff Member, Oak Ridge National Laboratory
Warren, Leonard, Professor of Therapeutic Research, University of Pennsylvania School of Medicine
Watanabe, Akira, National Institutes of Health
Webb, George D., Visiting Fellow, Columbia University, College of Physicians and Surgeons
Webb, H. Marguerite, Associate Professor of Biological Sciences, Goucher College and Northwestern University
Weiss, Leon, Associate Professor of Anatomy, The Johns Hopkins University
Werman, Robert, Professor of Psychiatry, Anatomy, and Physiology, Indiana University
Wiercinski, Floyd J., Professor, Illinois Teachers College North
Wilson, Walter L., Professor of Biology, Oakland University
Witkovsky, Paul, Assistant Professor of Physiology, Columbia University

Wyttenbach, Charles R., Assistant Professor of Anatomy, University of Chicago
Young, David K., Systematics-Ecology Program, Marine Biological Laboratory
Zigman, Seymour, Assistant Professor of Biochemistry, University of Rochester
Zinn, Donald J., Systematics-Ecology Program, Marine Biological Laboratory
Zullo, Victor A., Systematics-Ecology Program, Marine Biological Laboratory
Zwilling, Edgar, Professor of Biology, Brandeis University

Lalor Fellows, 1966

Michael Abercrombie, Senior Fellow, Head and Professor of Zoology, University College, London, England
Giovanni Giudice, University of Palermo, Italy
Antonio Giuditta, International Laboratory of Genetics and Biophysics, Naples, Italy
Allen W. Schuetz, University of Minnesota

Lillie Fellow, 1966

Clifford Grobstein, University of California, San Diego

Grass Fellows, 1966

Andrew F. Huxley, Senior Fellow, University College London, England
John David Birkhoff, Columbia University, College of Physicians and Surgeons
Joel E. Brown, Massachusetts Institute of Technology
Allen I. Selverston, University of Oregon

Rand Fellows, 1966

William T. W. Potts, University of Birmingham, England
Anthony O. W. Stretton, Harvard Medical School

Research Assistants, 1966

Abraham, Jerrold L., Massachusetts Institute of Technology
Anderson, Nels C., Jr., Duke University
Antonellis, Blenda C., Western Reserve University
Apley, Martyn L., Syracuse University
Appel, Antoinette R., Queens Campus of the City University of New York
Atkinson, Burr G., University of Connecticut
Audette, Valerie Anne, University of Vermont
Bailey, Claudia Frazier, Oberlin College
Baird, Spencer L., Oregon State University
Baker, Ailsie Fays, University of Texas
Bancroft, Frank Carter, University of California, Berkeley
Barnett, Gerald R., The Johns Hopkins School of Medicine
Barnhill, Robert, Miami University, Ohio
Barnwell, Franklin H., Northwestern University
Bartels, Eva, Columbia University, College of Physicians and Surgeons
Bjoraker, Barbara, University of Wisconsin
Bloedel, James, University of Minnesota
Border, Wayne, Washington University Medical School
Boyer, John F., University of Chicago
Brady, Francine, Syracuse University
Brander, Keith, Systematics-Ecology Program, Marine Biological Laboratory
Brown, Stephen C., University of Michigan
Burky, Albert J., Syracuse University
Burns, Susan, Systematics-Ecology Program, Marine Biological Laboratory
Carolan, Robert M., Harvard Medical School

Clark, John I., Dartmouth College
Cobb, William R., Systematics-Ecology Program, Marine Biological Laboratory
Collins, Sandra, University of Chicago
Cone, Margaret Virginia, University of Massachusetts
Conta, Barbara Ann, University of Michigan
Couch, Ernest F., Tulane University
Cox, Charles D., Systematics-Ecology Program, Marine Biological Laboratory
Czikowsky, Janice L., Systematics-Ecology Program, Marine Biological Laboratory
Dacey, Diane, Queen's University, Canada
Dendinger, Susan, College of St. Mary of the Springs
DePhillips, Henry A., Jr., Trinity College
DeSha, David L., Tulane University
DiCecco, Frank M., Washington University
Doane, Marshall, G., University of Maryland
Donley, Clark S., The Johns Hopkins University
Dyro, Frances Mary, National Institutes of Health
Eberhard, Anatol, Harvard University
Eberhard, Carolyn, Boston University
Eigner, Elizabeth Ann, University of New Mexico
Eisenberg, James P., Colby College
Emerson, Charles P., Jr., Massachusetts Institute of Technology
Emerson, Suzanne U., University of California, San Diego
Fass, Simcha, Massachusetts Institute of Technology
Findley, Davis, University of Tennessee
Fischer, Karen G., Harvard Medical School
Fishman, Harvey M., National Institutes of Health
Fitzjarrell, Austin T., Tulane University
Found, Bruce, Systematics-Ecology Program, Marine Biological Laboratory
Frick, Jane, Columbia University, College of Physicians and Surgeons
Fusco, Madeline, Womans Medical College
Gateff, Elisabeth, Western Reserve University
Gierty, John, Oakland University
Gittinger, John William, Oberlin College
Glick, John Harrison, Columbia University, College of Physicians and Surgeons
Granger, R. Eugene, Western Reserve University
Grebe, Stephen Charles, University of Pennsylvania
Greene, Douglas Alan, Princeton University
Gutman, George André, Columbia University
Hamby, Robert Jay, University of Chicago
Hamilton, John David, Princeton University
Hammerman, Kenneth J., New York University School of Medicine
Harris, Edward M., Duke University
Harosi, Ferenc, The Rockefeller University
Heller, James M., Systematics-Ecology Program, Marine Biological Laboratory
Heymann, Peter Walter, Washington University
Hinegardner, Ralph T., Columbia University
Hinsch, Gertrude W., Mt. Union College
Hofman, Florence, City College of New York
Huebner, Erwin, University of Massachusetts
Humphreys, Susie, Harvard University
Hunter, William Bruce, University of California, Berkeley
Irwin, M. Linda, Western Reserve University
Jared, Donald W., Oak Ridge National Laboratory
Kai Kong, Wong, Oberlin College
Karnofsky, Elisa B., Mt. Holyoke College
Kelenbeck, Edna K., Syracuse University
Kiester, Ross A., Systematics-Ecology Program, Marine Biological Laboratory
Kimball, Frances, Reed College

Kohne, David E., Carnegie Institution of Washington
Kornblith, Regina L., Brown University
Kourtz, Carol, Systematics-Ecology Program, Marine Biological Laboratory
Kratowich, Nancy R., Columbia University, College of Physicians and Surgeons
Krawchenko, John, Upstate Medical Center, at Syracuse
Kuppinger, Penny, Systematics-Ecology Program, Marine Biological Laboratory
Lange, Mary T., University of Pennsylvania School of Medicine
Larsen, William James, Western Reserve University
Lehman, William J., Princeton University
Leppla, Stephen H., University of Wisconsin
Levin, David Charles, Reed College
Lipson, Stephen Jay, Yale University
Livengood, David R., Indiana University
Macdonald, Victor W., The Johns Hopkins University
McCusker, Katherine, Dana Hall
McMahon, John J., Providence College
Meretsky, Dianne, Columbia University
Merkin, Terry E., University of Miami
Merrill, Charlotte, Massachusetts Institute of Technology
Mets, Laurens J., Pomona College
Mitchell, Beverly, Harvard Medical School
Moffett, David Franklin, North Carolina State University and Duke University
Montgomery, Mary Virginia, University of Connecticut
Mpitsos, George J., University of Virginia
Mullin, Kathleen P., Brown University
Murgola, Emanuel J., Yale University
Nadol, Joseph B., Jr., Harvard College
Nichols, T. Richard, Brown University
Nobel, Jack, University of New Mexico School of Medicine
Oldham, Peter J., Systematics-Ecology Program, Marine Biological Laboratory
Oselka, Janet, Columbia University
Ostergard, James P., Systematics-Ecology Program, Marine Biological Laboratory
Ott, Franklyn, Systematics-Ecology Program, Marine Biological Laboratory
Palmer, Betsy, Systematics-Ecology Program, Marine Biological Laboratory
Paranick, Joan Ann, Mellon Institute
Parsons, Maxine Frances, Memorial University of Newfoundland
Paynter, Emily, Brown University
Pederson, Judith B., Syracuse University
Pederson, Thoru, Syracuse University
Peters, Virginia B., New York University
Platt, Christopher, University of Chicago
Poluhowich, John J., University of Connecticut
Poston, R. N., University of Cambridge, England
Potter, Diane, Columbia University
Prusch, Robert, Syracuse University
Raab, Jacob L., University of Chicago
Raab, Margaret U., Massachusetts Institute of Technology
Ravitz, Melvyn Jay, Oakland University
Rhoades, Russell George, Kent State University
Rice, Virginia, Iowa State University
Richmond, Arthur P., Boston University
Robertson, Lola E., American Museum of Natural History
Rockford, Susan Jane, University of Rochester
Rome, Dan, Columbia University
Rueckert, Roland R., University of Wisconsin
Saltzman, Orah, Barnard College
Sameoto, Douglas, Queen's University, Canada
Sandlin, Ronald A., National Institutes of Health

Santos, Stuart L., Systematics-Ecology Program, Marine Biological Laboratory
Saul, David, The Johns Hopkins Medical School
Sawada, Nobuaki, Princeton University and Chiba University, Japan
Schearer, S. Bruce, Columbia University, College of Physicians and Surgeons
Schwamb, Peter E., Systematics-Ecology Program, Marine Biological Laboratory
Shapiro, Francis, Indiana University
Siegel, Barry W., Rensselaer Polytechnic Institute
Sindelar, William F., Western Reserve University
Sloane, Molla Rebecca, Wellesley College
Smarsh, Anne, VA Hospital, Boston
Smith, Barry Hamilton, Massachusetts Institute of Technology
Sommerfeld, Milton Ray, Washington University
Sorenson, Robert Lowell, University of Minnesota
Speel, Janis A., Systematics-Ecology Program, Marine Biological Laboratory
Spencer, Ann Gore, North Carolina State University
Spencer, Rebecca L., Columbia University
Squire, Richard D., North Carolina State University
Steen, Rebecca H., Yale University
Stool, Edward William, Tulane University
Surgenor, Peter Douglas, Hamilton College
Sweeney, A. Randolph, University of Illinois
Sylvia, Avis Latham, University of Connecticut
Taber, Robert L., University of Pittsburgh School of Medicine
Tabor, Gary L., New York University
Tannenbaum, Alice Susan, University of Maryland School of Medicine
Tashiro, Jay Shiro, Kenyon College
Teitelbaum, Mae, Systematics-Ecology Program, Marine Biological Laboratory
Terman, Stanley A., Massachusetts Institute of Technology
Thabes, Tamara M., University of Chicago
Thomas, Steven P., Indiana University
Tilney, Lewis G., Harvard University
Tokura, S., Northwestern University
Tuttle, Joan P., University of Rochester
Uhrich, John Frederick, Trinity University
Van Praag, Dina, New York University School of Medicine
Van Zandt, Dirk, Systematics-Ecology Program, Marine Biological Laboratory
Vargo, Diane M., University of Illinois
Vinson, Walter, Massachusetts Institute of Technology
Waldbaum, Elsa B., Swarthmore College
Wechsler, James A., Yale University
Weians, Patricia Katherine, College of St. Mary of the Springs
Weiner, Beverly, Harvard University
Werner, Irving, Brown University
Wertheimer, Michael D., Columbia University, College of Physicians and Surgeons
Wheeler, Richard F., Amateur Research Center, Inc.
White, Alan W., Systematics-Ecology Program, Marine Biological Laboratory
Wiksell, Audre Jean, Washington University
Williams, Madelaine M., American Museum of Natural History
Yuyama, Shuhei, Western Reserve University
Zelenka, Robert S., The Johns Hopkins University

Library Readers, 1966

Alscher, Ruth Paula, Professor of Biology, Manhattanville College
Ball, Eric G., Professor of Biochemistry, Harvard Medical School
Barlow, Grant H., Group Leader, Physical Biochemistry, Abbott Laboratories
Berne, Robert M., Professor of Physiology, Western Reserve University
Battelle Memorial Institute, William F. Clapp Laboratories, Inc.

Bersohn, Richard, Professor of Chemistry, Columbia University
Bodansky, Oscar, Chief, Division of Biochemistry, Sloan-Kettering Institute for Cancer Research
Bridgman, Anna Josephine, Chairman and Professor of Biology, Agnes Scott College
Buck, John B., Chief, Laboratory of Physical Biology, National Institutes of Health
Bunge, Richard, Assistant Professor of Anatomy, Columbia University
Butler, Elmer G., Professor of Biology, Princeton University
Carlson, Francis D., Chairman and Professor of Biophysics, The Johns Hopkins University
Chase, Aurin M., Professor of Biology, Princeton University
Child, Frank M., Associate Professor of Biology, Trinity College
Clark, Arnold M., Professor of Biological Sciences, University of Delaware
Cobb, Jewel Plummer, Executive Secretary of Science Department, Professor, Sarah Lawrence College
Cohen, Seymour S., Chairman and Professor of Therapeutic Research, University of Pennsylvania
Conover, Shirley M., Scholar, Radcliffe Institute for Independent Study
Crotty, W. J., Professor of Biology, New York University, Washington Square College
Edds, Mac V., Jr., Director of Medicine, Division of Biological and Medical Sciences, Brown University
Fine, Jacob, Professor of Surgery, Emeritus, Harvard Medical School
Flavin, Rev. John W., Chairman and Professor of Biology, College of the Holy Cross
Flesch, Peter, Research Professor of Dermatology, University of Pennsylvania
Gabriel, Mordecai L., Chairman and Professor of Biology, Brooklyn College, The City University of New York
Gitlin, David, Professor of Pediatrics, University of Pittsburgh School of Medicine
Gorlin, Richard, Assistant Professor of Medicine, Harvard Medical School
Green, James W., Professor of Physiology, Rutgers University
Green, Paul B., Associate Professor of Biology, University of Pennsylvania
Grobstein, Clifford, Chairman and Professor of Biology, University of California, San Diego
Gurewich, Vladimir, Assistant Professor of Clinical Medicine, New York College of Medicine
Handler, Philip, J. B. Duke Professor of Biochemistry, Duke University
Haubrich, Robert R., Associate Professor of Biology, Denison University
Hessler, Anita Y., Research Associate, Marine Biology Laboratory
Hiatt, Howard H., Professor of Medicine, Harvard Medical School
Holtzer, Howard, Director and Professor of Program in Cell Differentiation, University of Pennsylvania School of Medicine
Horovitz, Mark Willard, Biophysicist and Mathematical Programmer, University of California, Berkeley
Huritz, Jerard, Professor of Developmental Biology and Cancer, Albert Einstein College of Medicine
Isselbacher, Kurt J., Chief, Gastrointestinal Unit, Massachusetts General Hospital
Jaccarini, Victor, St. Aloysius' College, B'Kara Malta
Jacobs, M. H., Professor Emeritus, University of Pennsylvania
Kaltenbach, Jane Couffer, Associate Professor of Zoology, Mount Holyoke College
Keosian, John, Professor of Biology, Rutgers, The State University
Kopac, M. J., Professor of Biology, New York University, Washington Square College
Kowal, Jerome, Research Associate of Medicine, Mt. Sinai Hospital
Kravitz, Edward A., Assistant Professor of Neurophysiology and Neuropharmacology, Harvard Medical School
Leighton, Joseph, Professor of Pathology, University of Pittsburgh School of Medicine
Lennox, Edwin S., Senior Fellow, The Salk Institute for Biological Studies
Levinthal, Cyrus, Professor of Biophysics, Massachusetts Institute of Technology
Lin, Sheng H., Professor of Chemistry, Arizona State University
Lineaweaver, Thomas H., III, Woods Hole, Massachusetts
Malkiel, Saul, Research Associate, Children's Cancer Research Foundation, Inc.
Marks, Paul A., Associate Professor, Columbia University, College of Physicians and Surgeons
Mateyko, G. M., Professor of Biology, New York University, Washington Square College
McDonald, Sister Elizabeth Seton, Professor of Biology, College of St. Joseph on the Ohio

Nason, Alvin, Associate Director of McCollum-Pratt Institute, The Johns Hopkins University
Novikoff, Alex B., Research Professor, Albert Einstein College of Medicine
Perlmann, Gertrude E., Associate Professor, The Rockefeller University
Pinneo, Lawrence R., Research Associate and Assistant Professor of Psychology, Delta Regional Primate Research Center
Ray, Peter M., Professor of Biology, University of California, Santa Cruz
Recknagel, Richard O., Professor of Physiology, Western Reserve University
Rosenkranz, Herbert S., Associate Professor of Microbiology, Columbia University, College of Physicians and Surgeons
Roth, Jay S., Professor of Zoology, University of Connecticut
Rowland, Lewis P., Associate Professor of Neurology, Columbia University, College of Physicians and Surgeons
Rudzinska, Maria A., Associate Professor, The Rockefeller University
Rugh, Roberts, Associate Professor of Radiology, Columbia University, College of Physicians and Surgeons
Russell, Henry D., Dover, Massachusetts
Steinhardt, Jacinto, Professor of Chemistry, Georgetown University
Stetten, DeWitt, Jr., Dean and Professor of Medicine, Rutgers Medical School
Stetten, Marjorie R., Research Professor of Experimental Medicine, Rutgers Medical School
Tweedell, Kenyon S., Associate Professor of Biology, University of Notre Dame
Wainio, Walter, Professor of Biochemistry, Rutgers, The State University of New Jersey
Waksman, Byron Halsted, Chairman, Department of Microbiology, Yale University
Wheeler, George E., Associate Professor of Biology, Brooklyn College
Wichterman, Ralph, Professor of Biology, Temple University
Wilber, Charles G., Director, Marine Laboratories, University of Delaware
Wilson, Thomas Hastings, Associate Professor of Physiology, Harvard Medical School
Yntema, Chester L., Professor of Anatomy, State University of New York, Upstate Medical Center, at Syracuse

Students, 1966

All students listed completed the formal course program, June 13–July 23. Asterisk indicates students completing post-course research program, July 24–September 3.

ECOLOGY

Aldous, Mary J., Dalhousie University
*Brody, Robert W., Columbia University
*Coskren, Thomas D., California Institute of Technology
*Dow, David D., Drexel Institute of Technology
*Finnerty, Mary A., College of New Rochelle
*Fowler, Susan, University of New Hampshire
Gelband, Gail S., City College of New York
*Johnson, Gerald F., Yale University
Joseph, Virginia E., Yale University
Labavitch, John M., Wabash College
*Lubin, Yael D., Columbia University
*McDaniel, Susan J. G., University of Oklahoma
*Miller, Michael C., Indiana University
*Mozley, Samuel C., Emory University
O'Brien, William J., Cornell University
Oldaker, Warren H., Dept. of Health, Education, & Welfare, U. S. P. H. S.
*Richerson, Peter J., University of California, Davis
*Rutherford, John G., Jr., Syracuse University
*Schuetz, Joan M.
Smith, Daniel P., Massachusetts Institute of Technology

EMBRYOLOGY

*Chamberlain, John P., University of Miami
Colquhoun, William R., The Rockefeller University
*DeCosse, Jerome J., Upstate Medical Center
*Dickinson, William J., The Johns Hopkins University
*Gamow, Elfriede, University of Colorado
*Gordon, Joel S., University of Pennsylvania
*Goudsmit, Esther, National Institutes of Health
*Haynes, John K., Jr., Brown University
*Hecht, Norman B., University of Illinois
Hingson, Dickson J., Harvard University
*Horn, Edward G., Princeton University
*Jeffrey, John J., Jr., Harvard Medical School and Massachusetts General Hospital
*Kucinski, Helen M., University of Massachusetts
*Lombardi, Donald P., Massachusetts Institute of Technology
*Namenwirth, Marion, Indiana University
*Robinson, Helen L., Bryn Mawr College
*Rouslin, William, University of Connecticut
*Silva, Wilma W., University of California, Berkeley
*Stackpole, Christopher W., Tulane University
*Whitt, Gregory S., Yale University

MARINE BOTANY

*Ballenger, Janice M., Albion College
*Blankley, William F., University of California, San Diego
*Brooks, Robert C., University of Chicago Graduate School
*Gallant, Esther M., Ohio Wesleyan University
Hambrick, Nancy A., Berry College
Hollick, B. Michael, Syracuse University
Jenkins, Hugh E., Oberlin College
*Leo, John P., Jr., Drew University
McCarthy, Jean C., University of Vermont
*Parker, Carol A., University of Massachusetts
de Parodi, Alicia, Instituto Pedagogico
*Pessoney, George F., University of Texas
*Pike, John D., University of Tennessee
Poyton, Robert Oliver, University of California, Berkeley
Rhee, Cornelis, Harvard University
*Scott, Joseph L., University of California, Santa Barbara
Specht, David T., Western State College of Colorado
*Waaland, Joseph R., University of California, Berkeley
Wells, Charles V., University of Arizona

PHYSIOLOGY

*Barclay, Nora E., University of Wisconsin
Bland, John H., University of Vermont
*Bryan, Joseph, University of Pennsylvania
*Burke, Charles R., University of Illinois
*Burt, David R., The Johns Hopkins University
*Cantor, Kenneth P., University of California, Berkeley
Clark, Benton C., Columbia University
Daigneault, Rejean, University of Montreal
*Essenberg, Richard C., Harvard University
*Forman, David S., The Rockefeller University

*Fowler, Elizabeth, Harvard University
Gerbi, Susan A., Yale University
Goel, Narendra S., University of Maryland
Janin, Joel M. C., Centre National de la Recherche Scientifique
Kottke, Margaret E., Brandeis University
Lieb, William R., University of Illinois
*Mitchell, George W., III, University of Illinois
*Muller, Kenneth J., University of Chicago
*Perkins, John H., Harvard University
Rogers, Lesley Joy, Harvard University
*Schloerb, Paul R., University of Kansas
Schnitzer, Thomas J., Princeton University
*Sesnowitz, Suzanne, Yale University
*Sterzing, Peter R., Iowa State University
*Sweetman, Lawrence, University of Miami
*Terwilliger, Robert C., Jr., Boston University
Widmayer, Dorothea J., Wellesley College
*Zelenka, Peggy Sue, The Johns Hopkins University

INVERTEBRATE ZOOLOGY

Avolizi, Robert J., Syracuse University
Bagus, Eileen D., Seton Hill College
Baird, Ronald C., Harvard University
Bierle, Donald A., University of South Dakota
Bosch, Herman F., The Johns Hopkins University
*Bouffard, Thomas G., Fordham University
Brown, Patricia S., University of Michigan
Cunnigen, Oliver W., III, Tougaloo College
Daniels, Mathew P., University of Chicago
Dresden, Marc H., Harvard University
Friend, Judith, Harvard University
Gavin, Sister Mary Lua, Mount St. Vincent College
*Geens, Maura, University of Delaware
Gwadz, Robert W., University of Notre Dame
Hill, Richard W., University of Michigan
*James, Margaret T., Duke University
Kien, Margaretha, Boston University
Kiester, Alan R., University of California, Berkeley
*Mattice, Jack S., Syracuse University
McDonald, Ruth I., Indiana University
McKenzie, Norma Dee A., University of Oregon
*Mesibov, Robert E., New York University
Nagin, Richard D., The Rockefeller University
*Olshan, Arthur R., Brooklyn College
Ramamurthy, Gollu V., University of California, Berkeley
*Ritch, Robert H., Harvard University
Schulman, Benita, Harvard University
Selzer, Gerald B., University of Oregon
*Simson, Jo Anne, State University of New York
Stanley, Anita G., Drew University
Stetten, Gail, Jameson A. Douglass College
Stiles, Sheila S., U. S. Bureau of Commercial Fisheries
*Stokes, Darrell R., University of Hawaii
Weare, Nancy M., The Johns Hopkins University
Woodin, Sarah A., Goucher College

Wyse, Beatrice M., Oregon State University
Zalon, Paul S., New York University

4. Fellowships and Scholarships, 1966

Bio Club Scholarship:
Gail S. Gelband, Ecology Course

The Father Arsenius Boyer Fellowship:
Rev. Victor Jaccarini, Library reader

The Gary N. Calkins Memorial Scholarship:
Darrell Robert Stokes, Invertebrate Zoology Course

The Turtox-Croasdale Scholarship:
Mary Esther Gallant, Botany Course

The Lucretia Crocker Scholarship:
Mary J. Aldous, Ecology Course
Janice Mae Ballenger, Botany Course

The Merkel H. Jacobs Scholarship:
Narendra Goel, Physiology Course

5. Training Programs

Fertilization and Gamete Physiology Training Program

I. Instructors

Charles B. Metz, University of Miami, in charge of program
C. R. Austin, Tulane University, Delta Regional Primate Research Center
Luther E. Franklin, Tulane University, Delta Regional Primate Research Center
Giovanni Giudice, University of Palermo, Italy
H. F. Linskens, University of Nijmegen, The Netherlands
Alberto Monroy, University of Palermo, Italy
Leonard Nelson, Emory University

II. Trainees

Alvin Bicker, New York University
Constante Ceccarini, University of Palermo, Italy
Barbara A. Cocanour, University of Maine
Sharon L. Desborough, University of Wisconsin
Ernest F. DuBrul, Washington University
Kenneth D. Graziano, The Johns Hopkins University
Kenneth W. Gregg, Emory University
Robert G. Hart, University of Illinois
Paul M. Heidger, Jr., Tulane University
Lawrence D. Koehler, Central Michigan University
Frank J. Longo, Oregon State University
Ronald J. Pfohl, Michigan State University
Allen H. Repkin, Emory University
Willie R. Smith, Long Island University
Richard C. Weisenberg, University of Chicago
David G. Whittingham, University of Pennsylvania and The Johns Hopkins University

III. LECTURES

GIOVANNI GIUDICE	Ribosomes in sea urchin embryos
ARTHUR COLWIN AND LAURA COLWIN	Early stages of fertilization: Sperm-egg relationships
LEONARD WARREN	The surface membranes of animal cells
ARTHUR ZIMMERMAN	Physical factors affecting cell division in marine eggs
WALTER TROLL	Effects of carcinogens and mutagens on DNA
EMIL STEINBERGER	Initiation and maintenance of spermatogenesis in the rat, *in vitro* and *in vivo*
MELVIN SPIEGEL	Enzyme regulation in amphibian development
R. G. WALES	Some recent studies on the metabolism of mammalian spermatozoa
H. F. LINSKENS	Biochemical background of the incompatibility barrier in fertilization of higher plants
LIONEL REBHUN	Assembly and disassembly of the spindle in marine eggs: *In vivo* and *in vitro* manipulation
W. K. WHITTEN	Pheromones and mammalian reproduction
LAUREL GLASS	Maternal molecular contributions to mamalian oocytes and early embryos

NEUROPHYSIOLOGY TRAINING PROGRAM

I. INSTRUCTORS

STEPHEN W. KUFFLER, Harvard Medical School, in charge of program
EDWIN J. FURSHPAN, Harvard Medical School
DAVID D. POTTER, Harvard Medical School

II. RESEARCH ASSOCIATE

ROBERT B. BOSLER, Harvard Medical School

III. ASSISTANT

MONROE COHEN, Harvard Medical School

IV. TRAINEES

THOMAS BENJAMIN, The Salk Institute for Biological Studies
SEYMOUR BENZER, California Institute of Technology
LAWRENCE COHEN, Columbia University
DAVID LANDOWNE, Harvard University
DONALD PFAFF, Massachusetts Institute of Technology
NICHOLAS SPITZER, Harvard Medical School
WILLIAM STELL, University of Chicago

COMPARATIVE PHYSIOLOGY RESEARCH TRAINING PROGRAM

I. INSTRUCTORS

LEWIS H. KLEINHOLZ, Reed College, in charge of program
BERNARD C. ABBOTT, University of Illinois
WILLIAM T. W. POTTS, University of Birmingham, England

II. RESEARCH ASSOCIATE

Y. MATSUMOTO, University of Illinois

III. ASSISTANT

FRANCES KIMBALL, Reed College

IV. TRAINEES

VALERIS ERNST, University of Louisville
JOHN O'BENAR, University of Illinois
PAUL PATTERSON, The Johns Hopkins University
MORRIS SELIGMAN, University of Illinois
JOHN STANGEL, New York Medical College
WILLIAM VAUGHAN, University of Illinois

V. LECTURES

L. H. KLEINHOLZ	Separation, purification and physiology of neurosecretory hormones
M. FINGERMAN	Chromatophores
F. A. BELAMARICH	Physiology and chemistry of pericardial organs
F. M. FISHER, JR.	Physiological aspects of parasitism
E. J. W. BARRINGTON	Endostyle of protochordates
F. A. BROWN, JR.	Biological clocks
W. T. W. POTTS	Physiology of osmoregulation
B. C. ABBOTT	Control of contraction and motility
D. E. COPELAND	Fine structure of salt-mobilizing tissues
R. RIKMANSPOEL	Elastic properties of flagellum in sperm
B. A. TWAROG	Catch mechanism in molluscan muscle
C. L. PROSSER	Mechanisms of smooth muscle contraction

6. TABULAR VIEW OF ATTENDANCE, 1962–1966

	1962	*1963*	*1964*	*1965*	*1966*
INVESTIGATORS—TOTAL	494	490	512	572	555
Independent	279	261	273	284	287
Library Readers	56	51	47	62	77
Research Assistants	159	178	192	227	191
STUDENTS—TOTAL	121	124	126	128	126
Invertebrate Zoology	38	40	40	41	37
Embryology	20	20	20	20	22
Physiology	28	28	30	30	29
Botany	20	20	19	20	18
Ecology	15	16	17	17	20
TRAINEES—TOTAL			30	34	29
Nerve-Muscle			7	7	7
Comparative Physiology			7	11	6
Fertilization & Gamete			16	16	16
TOTAL ATTENDANCE	615	614	668	734	710
Less persons represented in two categories	4	5	7	4	0
	611	609	661	730	710
INSTITUTIONS REPRESENTED—TOTAL	118	120	140	218	198
By Investigators	81	83	117	142	105
By Students	57	73	23	76	76
By Library Readers					47
By Research Assistants					80
FOREIGN INSTITUTIONS REPRESENTED	31	21	32	27	28
By Investigators	17	15	28	25	20
By Students	14	6	4	2	4
By Library Readers					1
By Research Assistants					4

7. Institutions Represented, 1966

Abbott Laboratories
Agnes Scott College
Albert Einstein College of Medicine
Albion College
Amateur Research Center, Inc.
American Museum of Natural History
Arizona State University
Arizona, University of
Barnard College
Battelle Memorial Institute
Berry College
Boston University
Boston University Graduate School
Bowdoin College
Brandeis University
Brooklyn College, City University of New York
Brown University
Bryn Mawr College
California Institute of Technology
California State College, at Los Angeles
California, University of, Berkeley
California, University of, Davis
California, University of, Irvine
California, University of, San Diego
California, University of, Santa Barbara
California, University of, Santa Cruz
Carnegie Institution of Washington
Central Michigan University
Chicago, University of
Chicago, University of, Graduate School
Children's Cancer Research Foundation, Inc.
Cincinnati, University of
Colby College
Colorado, University of
Columbia University
Columbia University, College of Physicians and Surgeons
Connecticut, University of
Connecticut, University of, School of Dental Medicine
Cornell University
Cornell University Medical School
Dana Hall
Dartmouth College
Dartmouth College Medical School
Delaware, University of
Delta Regional Primate Research Center, Tulane University
Denison University
Drew University
Drexel Institute of Technology
Duke University
Earlham College
Emory University
Florida Presbyterian College
Florida, University of, College of Medicine
Fordham University
Georgetown University
Goucher College
Hamilton College
Harvard College
Harvard Medical School
Harvard University
Harvard University Graduate School
Hawaii, University of
Holy Cross, College of the
Illinois Teachers College North
Illinois, University of
Indiana University
Indiana University Medical School
Institute for Cancer Research, The
Institute for Muscle Research, The
Iowa State University
Jameson A. Douglass College
Johns Hopkins University, The
Johns Hopkins University School of Hygiene & Public Health, The
Johns Hopkins University School of Medicine, The
Kansas, University of
Kent State University
Kenyon College
Lawrence Radiation Laboratory
Long Island University
Louisville, University of
Maine, University of
Manhattanville College
Marine Biological Laboratory
Marquette University
Maryland, University of
Maryland, University of School of Medicine
Massachusetts General Hospital
Massachusetts Institute of Technology
Massachusetts, University of
Mellon Institute, The
Miami University at Ohio
Miami, University of
Miami, University of, Institute of Molecular Evolution
Michigan State University
Michigan, University of

Minnesota, University of
Missouri, University of
Mount Holyoke College
Mount St. Joseph on the Ohio, College of
Mount St. Vincent, College of
Mount Sinai Hospital
Mount Union College
National Institutes of Health
National Science Foundation
New Hampshire, University of
New Mexico, University of
New Mexico, University of, School of Medicine
New Rochelle, College of
New York, City College of
New York College of Medicine
New York, State University of, College of Medicine at Buffalo
New York, State University of, Upstate Medical Center at Syracuse
New York University
New York University College of Dentistry
New York University Medical School
New York University, Washington Square College
North Carolina State University, at Raleigh
North Carolina, University of, at Chapel Hill
Northwestern University
Notre Dame, University of
Oakland University
Oak Ridge National Laboratory
Oberlin College
Ohio Wesleyan University
Oklahoma, University of
Oregon State University
Oregon, University of
Pennsylvania State University
Pennsylvania, University of
Pennsylvania, University of, School of Medicine
Pittsburgh, University of
Pittsburgh, University of, School of Medicine
Pomona College
Princeton University
Providence College
Purdue University
Queens College, City University of New York
Radcliffe College
Radcliffe Institute for Independent Study
Reed College
Rensselaer Polytechnic Institute
Rhode Island, University of
Rice University
Rochester, University of
Rochester, University of, School of Medicine and Dentistry
Rockefeller University, The
Russell Sage College
Rutgers Medical School
Rutgers, The State University of New Jersey
Salk Institute for Biological Studies, The
Sarah Lawrence College
Seton Hill College
Simmons College
Single Cell Research Foundation, Inc.
Sloan-Kettering Institute for Cancer Research
Smithsonian Institution
South Dakota, University of
St. Mary of the Springs, College of
Stanford University School of Medicine
Swarthmore College
Syracuse University
Temple University
Tennessee, University of
Texas, University of
Thayer Academy
Tougaloo College
Trinity College
Trinity University
Tulane University
U. S. Army Natick Laboratories
U. S. Bureau of Commercial Fisheries
U. S. Department of Health, Education, & Welfare, Public Health Service
Vassar College
Vermont, University of
Veterans Administration Hospital, Boston
Veterans Administration Hospital, Brooklyn
Veterans Administration Hospital, Pittsburgh
Virginia, University of
Wabash College
Wake Forest College
Washington University
Washington University School of Medicine
Washington, University of
Wellesley College
Wesleyan University
West Florida, University of
Western Reserve University
Western Reserve University Medical School
Western State College of Colorado
William and Mary, College of
Wilson College
Wisconsin, University of
Women's Medical College of Pennsylvania
Woods Hole Oceanographic Institution
Yale University
Yale University School of Medicine

Foreign Institutions Represented, 1966

Biologische Anstalt Helgoland, West Germany
Birmingham, University of, England
Bristol, University of, England
British Columbia, University of, Canada
Cambridge, University of, England
Centre National de la Recherche Scientifique
Chiba University, Japan
Dalhousie University, Nova Scotia
Dalhousie University Medical School, Nova Scotia
Genoa, University of, Italy
Glasgow, University of, Scotland
Instituto Pedagogio, Venezuela
International Laboratory of Genetics & Biophysics, Italy
Israel Institute of Biological Research, Ness Ziona
Leeds, University of, England
Leicester, University of, England
Ljubljana, University of, Yugoslavia
Memorial University of Newfoundland, Canada
Montreal University, Canada
Nijmegen, University of, The Netherlands
Ottawa, University of, Canada
Pahlavi University, Shiraz, Iran
Palermo, University of, Italy
Queen's University, Canada
St. Aloysius' College, Malta
Teheran University, Iran
Tohoku University, Japan
University College London, England

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Office of Naval Research
Mrs. Arthur Kemble Parpart
The Rockefeller University
Gerard Swope, Jr.
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James H. Wickersham
Whitehall Foundation, Inc.

8. Friday Evening Lectures, 1966

July 1

David L. Nanney .... Corticotype Transmission in *Tetrahymena*
University of Illinois

July 8

Edwin J. Furshpan .... Specialized Intercommunications Between Cells in Embryos and Tissue Culture
Harvard Medical School

July 14

Andrew F. Huxley .... On the Assumption of Uniformity in Nature
University College London
Alexander Forbes Lecturer at the MBL

July 15

Andrew F. Huxley .... The Contractile Process in Striated Muscle

July 22

Maurice Sussman .... Temporal Control of Genetic Transcription and Translation during Slime Mold Development
Brandeis University

July 29

Robert A. Good University of Minnesota Medical School	The Development of Immunological Capacity (Cancelled)

August 5

LaMont C. Cole Cornell University	The Quantitative Approach to Ecological Problems

August 12

Michael Abercrombie University College London Senior Lalor Fellow at the MBL	Cancer as a Disorder of the Cell Surface

August 19

David J. L. Luck The Rockefeller University	Formation of Mitochondria in Growing Cells

August 26

Clifford Grobstein University of California, San Diego	Beyond Heredity

9. Tuesday Evening Seminars, 1966

July 5

W. A. H. Rushton	S-Potentials from Fish Retina: The Relation to Light Intensity and Area
H. Bernard Hartman	An Electrophysiological Investigation Relating the Structure and Function of the Crustacean Proprioceptor, the Propus-Dactylus Organ
Walter C. Vinson	Entry of mRNA-Ribosomal Subunit Particles into Cytoplasmic Polyribosomes during Early Sea Urchin Embryogenesis

July 26

George Szabó	Racial Coloration and Suntanning in Man
Lewis G. Tilney John R. Gibbins	Microtubules and Morphogenesis. The Role of Microtubules in the Development of the Primary Mesenchyme in the Sea Urchin Embryo
Walter Auclair Barry Siegel	Cilia Regeneration in the Sea Urchin Embryo

August 9

D. R. Shanklin	The Effect of 15° C. on the Stages of Normal Development of *Fundulus heteroclitus*
D. R. Shanklin	Rate of Hatching of *Fundulus heteroclitus* at 20° C. and the effect of Prior Exposure at 15° C.
J. P. Revel M. Karnovsky	Fine Structure of Tight Junctions
A. J. D. de Lorenzo	Structure and Function of the Synaptic Junctions of the Chick Ciliary Ganglion
G. D. Pappas M. V. L. Bennett	Fine Structure of Neuronal Junctions Involved in Different Transmission Systems in the CNS

August 16

Andrew Hegyeli	Chemical Studies of Directin
Ruth Johnson-Hegyeli Andrew Hegyeli	*In vitro* and *in vivo* Studies of Directin
Joseph P. Senft	The Effects of some Inhibitors on the Temperature-Dependent Component of the Lobster Axon Resting Potential
R. E. Stephens R. E. Kane	Studies on a Major Protein from Isolated Sea Urchin Egg Cortex

10. Members of the Corporation, 1966

Including Action of 1966 Annual Meeting

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Bevelander, Dr. Gerrit, Dental Branch, Medical Center, University of Texas, Houston, Texas 77025

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Carpenter, Dr. Russell L., Tufts University, Medford, Massachusetts 02155

Carriker, Dr. Melbourne R., Systematics-Ecology Program, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

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Chaet, Dr. Alfred B., University of West Florida, Pensacola, Florida 32505

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Cleland, Dr. Ralph E., Department of Botany, Indiana University, Bloomington, Indiana 47401

Clement, Dr. A. C., Department of Biology, Emory University, Atlanta, Georgia 30322

Cohen, Dr. Seymour S., Department of Therapeutic Research, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

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Colton, Dr. H. S., P. O. Box 699, Flagstaff, Arizona 86001

Colwin, Dr. Arthur L., Department of Biology, Queens College, Flushing, New York 11367

Colwin, Dr. Laura H., Department of Biology, Queens College, Flushing, New York 11367

Cooper, Dr. Kenneth W., Department of Cytology, Dartmouth Medical School, Hanover, New Hampshire 03755

Cooperstein, Dr. Sherwin J., School of Dental Medicine, University of Connecticut, Hartford, Connecticut 06105

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Crane, Mr. John O., Woods Hole, Massachusetts 02543

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Croasdale, Dr. Hannah T., Dartmouth College, Hanover, New Hampshire 03755

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Dan, Dr. Katsuma, Misaki Biological Station, Misaki, Japan

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Weiss, Dr. Paul A., The Rockefeller University, New York, New York 10021

Wenrich, Dr. D. H., Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104

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Whiting, Dr. Phineas, 535 West Vanderbilt Drive, Oak Ridge, Tennessee 37830

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Zorzoli, Dr. Anita, Department of Physiology, Vassar College, Poughkeepsie, New York 12601

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Zwilling, Dr. Edgar, Department of Biology, Brandeis University, Waltham, Massachusetts 02154

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Crane, Miss Louise
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Crossley, Mr. and Mrs. Archibald M.
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Hervey, Mrs. John P.
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Mitchell, Mrs. Philip
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Newton, Miss Helen K.
Nichols, Mrs. George
The Aaron E. Norman Fund, Inc.
Packard, Mrs. Charles
Parpart, Mrs. Arthur K.
Park, Mr. Malcolm S.
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Riggs, Mr. and Mrs. Lawrason, III
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Rogers, Mrs. Charles E.
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Schwartz, Mrs. Victor B.
Shiverick, Mrs. Arthur
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Stone, Dr. and Mrs. William, Jr.
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Stunkard, Mrs. Horace
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Swope, Mr. David
Swope, Mr. and Mrs. Gerard, Jr.
Swope, Miss Henrietta H.
Szent-Györgyi, Dr. Albert
Tompkins, Mr. and Mrs. B. A.
Warren, Dr. and Mrs. Shields
Webster, Mrs. Edwin S.
Whiteley, Miss Mabel W.
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Whiting, Dr. and Mrs. Phineas W.
Whitney, Mrs. George
Wickersham, Mrs. James H.
Wichterman, Mrs. Ralph
Wilhelm, Dr. Hazel S.
Wilson, Mrs. Edmund B.
Wilson, Dr. May G.
Winters, Dr. Robert W.
Wolfe, Dr. Charles
Wolfinsohn, Mrs. Wolfe
Wrinch, Dr. Dorothy
Yntema, Mrs. Chester L.

V. REPORT OF THE LIBRARIAN

The major project in the Library this year was the changeover to the Library of Congress system of classifying books. Approximately 15,000 volumes in the book section were re-classified by subject and new, more readable labels were applied to the books. The change was completed before the summer season started so the confusion was kept to a minimum. The alphabetical system of shelving journals was not changed in any way.

Inter-library loan requests from universities, government agencies and industry were doubled over the previous year. The Library received and processed 2,850 individual requests for articles held here in Woods Hole. We made 241 requests for articles from other libraries for the use of investigators here.

Physical changes were made on the reprint floor and the main reading room. During the winter months we changed from the numbering system on reprints to filing strictly by author. All boxes have now been relabeled to list the contents by author rather than number, thereby making it easier to locate needed reprints and also eliminating a great deal of detailed work for the staff. Reprints are now added to the collection shortly after they are received in the Library.

The main reading room was refurnished with new chairs, and more open racks were added for display of current volumes. Two hundred and fifty additional journal titles are now displayed in this room due to the new rack space. The eight carrells and typing room on the third floor were completed and used during the summer months.

Nearly 2,000 volumes were sent to the bindery in 1966 and the total holdings are now 129,766. This figure does not include the reprint floor.

Total number of serial titles in library			3,874
Number received currently			2,204
On subscription	873		
On exchange	930		
On gift basis	320		
Number of reference books added in 1966		481	
Received from book exhibitors		108	
Total number of reprints in collection			238,220
Number added in 1966			2,557

Respectfully submitted,
JANE FESSENDEN,
Librarian

VI. REPORT OF THE TREASURER

The market value of the general Endowment Fund and the Library Fund at December 31, 1966, amounted to $2,249,448, as against book value of $1,326,668. This compares with values of $2,502,903 and $1,261,842, respectively, at the end of the preceding year. The average yield on the securities as 3.9% of the market value and 6.6% of the book value. Uninvested principal cash in the above amounts at the end of the year was $1,105. Classification of the securities held in the Endowment Fund appears in the Auditor's Report.

The market value of the pooled securities as of December 31, 1966, was $701,945, as compared with $640,065, being the market value as of December 31, 1965. Uninvested principal cash at the end of the year was $21,289.85. Book value of securities in this account at the beginning of this year was $528,319, compared with $664,523, at the close of 1966. The increase being the result of additional funds received from Herbert W. Rand Fellowships, Mary Rogick Fund and Mr. and Mrs. Gerard Swope, Jr. The average yield on market value was 3.3% and 3.5% on book value.

The proportionate interest in the Pool Fund Account of the various Funds, as of December 31, 1966, is as follows:

Pension Funds	17.055%
General Laboratory Investment	24.860%
F. R. Lillie Memorial Fund	11.364%
Anonymous Gift	.648%
Other:	
Bio Club Scholarship Fund	.542%
Rev. Arsenius Boyer Scholarship Fund	.823%
Gary N. Calkins Fund	.679%
Allen R. Memhard Fund	.167%
Lucretia Crocker Fund	1.902%
E. G. Conklin Fund	.398%
Jewett Memorial Fund	.247%
M. H. Jacobs Scholarship Fund	.308%
Herbert W. Rand Fellowship	26.455%
Mellon Foundation	6.344%
Mary Rogick Fund	2.520%
Swope Foundation	5.688%

Donations from the MBL Associates for 1966 were $7,595.00 as compared with $7,385.00 for 1965. Unrestricted gifts from foundations, societies and companies amounted to $17,125.

During the year, we administered the following grants:

Investigators	*Training*	*MBL Institutional*
12 NIH	3 NIH	3 NIH
6 NSF	2 NSF	2 NSF
1 Ford		1 Ford
1 Whitehall		2 ONR
2 ONR		1 AEC
—	—	—
22	5	9

The rate of overhead on grants to investigators is 20% based on the amount expended. However, for two NIH grants the allowance for overhead costs was not awarded until after the end of the year, and for one NSF grant, the overhead allowance has not yet been awarded, pending completion of negotiation of an indirect cost rate. The overhead on those grants wherein provision therefore was made in the funds awarded within the year, amounted to $85,822 as compared with $94,791 for the preceding year. A proposal to NIH for determining an indirect cost rate for the current year is in course of preparation.

The General Biological Supply House fiscal year ended June 30, 1966, and had a profit after taxes of $313,553 as compared to $275,080 in 1965, $309,651 in 1964, $241,616 in 1963, and $302,657 in 1962.

During the period covered by this report the Marine Biological Laboratory received dividends from the General Biological Supply House of $63,500 as against $63,500 in 1965, $63,500 in 1964, $42,164 in 1963, and $38,000 in 1962.

The following is a statement of the auditors:

To the Trustees of Marine Biological Laboratory, Woods Hole, Massachusetts:

We have examined the balance sheet of Marine Biological Laboratory as at December 31, 1966, the related statement of operating expenditures and income and statement of funds for the year then ended. Our examination was made in accordance with generally accepted auditing standards, and accordingly included such tests of the accounting records and such other auditing procedures as we considered necessary in the circumstances. We examined and have reported on financial statements of the Laboratory for the year ended December 31, 1965.

In our opinion, the accompanying financial statements present fairly the assets, liabilities and funds of Marine Biological Laboratory at December 31, 1966 and 1965, and the results of its operations for the years then ended on a consistent basis.

The supplementary schedules included in this report were obtained from the Laboratory's records in the course of our examination and, in our opinion, are fairly stated in all material respects in relation to the financial statements, taken as a whole.

Boston, Massachusetts
April 28, 1967 LYBRAND, ROSS BROS. AND MONTGOMERY

It will be noted from the operating statement that the Laboratory activities are running at a rate of about 1.6 million dollars per year. This is an increase of just over a half million since 1960.

ALEXANDER T. DAIGNAULT,
Treasurer

MARINE BIOLOGICAL LABORATORY

BALANCE SHEETS

December 31, 1966 and 1965

Investments	*1966*	*1965*
Investments held by Trustee:		
Securities, at cost (approximate market quotation 1966—$2,249,448)	$1,326,668	$1,261,842
Cash	1,105	1,878
	$1,327,773	$1,263,720
Investments of other endowment and unrestricted funds:		
Pooled investments, at cost (approximate market quotation 1966—$701,945) less $5,728 temporary investment of current fund cash	658,795	522,591
Other investments	121,370	119,352
Cash	50,108	55,608
Accounts receivable	1,393	7,384
	$2,159,439	$1,968,655
Plant Assets		
Land, buildings, library and equipment (note)	5,649,105	5,481,019
Less allowance for depreciation (note)	1,537,471	1,449,145
	4,111,634	4,031,874
Construction in progress	28,453	41,253
Cash	6,056	90,015
Short-term investments, at cost	50,000	50,000
	$4,196,143	$4,213,142
Current Assets		
Cash	48,860	35,596
Temporary investment in pooled securities	5,728	5,728
U. S. Treasury bills, at cost	58,219	74,210
Accounts receivable (U. S. Government, 1966—$55,247; 1965—$78,108)	127,817	139,079
Inventories of supplies and Bulletins	40,525	35,493
Other assets	31,580	1,893
	$ 312,729	$ 291,999

Note—The Laboratory has since January 1, 1916 provided for reduction of book amounts of plant assets and funds invested in plant at annual rates ranging from 1% to 5% of the original cost of the assets.

MARINE BIOLOGICAL LABORATORY

BALANCE SHEETS

December 31, 1966 and 1965

Invested Funds

	1966	*1965*
Endowment funds given in trust for benefit of the Marine Biological Laboratory	$1,327,773	$1,263,720
Endowment funds for awards and scholarships:		
Principal	427,663	322,135
Unexpended income	24,297	24,400
	451,960	346,535
Unrestricted funds functioning as endowment	206,378	206,378
Retirement fund	150,703	135,952
Pooled investments—accumulated gain	22,625	16,070
	$2,159,439	$1,968,655
Plant Funds		
Funds expended for plant, less retirements	$5,677,558	$5,522,272
Less allowance for depreciation charged thereto	1,537,471	1,449,145
	4,140,087	4,073,127
Unexpended plant funds	56,056	140,015
	$4,196,143	$4,213,142
Current Liabilities and Funds		
Accounts payable and accrued expenses	$ 64,385	53,563
Advance subscriptions	16,746	16,074
Unexpended grants—research	61,780	64,094
Unexpended balances of gifts for designated purposes	22,281	18,772
Current fund	142,537	139,496
	$ 312,729	$ 291,999

MARINE BIOLOGICAL LABORATORY

Statements of Operating Expenditures and Income

Years Ended December 31, 1966 and 1965

Operating Expenditures

	1966	*1965*
Research and accessory services	$ 329,066	$ 294,798
Instruction	179,964	196,509
Library and publications (including book purchases—1966, $34,082; 1965, $39,356)	105,857	102,595
Direct costs on research grants	466,824	514,709
Direct costs on institution support grants	110,993	161,669
	$1,192,704	$1,270,280
Administration and general	139,888	125,298
Plant operation and maintenance	147,271	131,885
Dormitories and dining	198,668	193,298
Additions to plant from current fund	34,528	128,448
	$1,713,059	$1,849,209
Less depreciation included in plant operation and dormitories and dining above but charged to plant funds	88,786	71,461
	$1,624,273	$1,777,748

Income

Research fees	97,186	96,745
Accessory services (including sales of biological specimens—1966, $34,551; 1965, $37,586)	133,416	107,204
Instruction fees	28,750	28,525
Library fees, Bulletins, subscriptions and other	64,705	53,687
Dormitories and dining income	141,393	149,754
Grants for support of institutional activities:		
Instruction and training	177,825	198,404
Support services	110,993	161,669
General	130,750	106,445
Reimbursements and allowances for direct and indirect costs on specific research grants	534,909	592,225
Gifts used for current expenses	17,125	66,368
Investment income used for current expenses	162,262	157,310
	$1,599,314	$1,718,336
Excess current income (expenditures)	($ 24,959)	($ 59,412)

MARINE BIOLOGICAL LABORATORY

STATEMENT OF FUNDS

Year Ended December 31, 1966

	Balance December 31, 1965	*Gifts and Other Receipts*	*Investment Income*	*Used for Current Expenses*	*Other Expenditures*	*Balance December 31, 1966*
Invested funds	$1,968,655	$ 199,825	$175,863	$ 158,902	$ 26,002	$2,159,439
Unexpended plant funds	$ 140,015		3,241		87,200	$ 56,056
Unexpended research grants	$ 64,094	952,163		954,477		$ 61,780
Unexpended gifts for designated purposes	$ 18,772	25,023		17,125	4,389	$ 22,281
Current fund	$ 139,496	(24,959) (1) 28,000 (2)				$ 142,537
		$1,180.052	$179,104	$1,130,504	$117,591	
Gifts		130,698				
Grants for research, training and support		952,163				
Appropriated from current income and other		23,689				
Net gain on sale of securities		70,461				
(1) Excess of current expenditures over income		(24,959)				
(2) Gift of boat (appraisal value) .		28,000				
		$1,180,052				
Expended for construction of boat					87,200	
Scholarship awards ...					11,908	
Payments to pensioners					14,094	
Other					4,389	
					$117,591	

MARINE BIOLOGICAL LABORATORY

SUMMARY OF INVESTMENTS

December 31, 1966

	Cost	*% of Total*	*Market Quotations*	*% of Total*	*Investment Income 1966*
Securities held by Trustee:					
General endowment fund:					
U. S. Government securities	$ 25,065	2.2	$ 25,156	1.4	$ 2,798
Corporate bonds	679,226	61.4	589,206	31.9	27,325
Preferred stocks	54,422	4.9	55,375	3.0	2,300
Common stocks	348,595	31.5	1,176,351	63.7	40,569
	1,107,308	100.0	1,846,088	100.0	72,992
General Education Board endowment fund:					
U. S. Government securities	67,074	30.6	66,841	16.6	2,524
Other bonds	98,996	45.1	87,310	21.6	4,445
Preferred stocks	5,141	2.3	4,388	1.1	250
Common stocks	48,149	22.0	244,821	60.7	7,639
	219,360	100.0	403,360	100.0	14,858
Total securities held by Trustee	$1,326,668		$2,249,448		$ 87,850
Investments of other endowment and unrestricted funds:					
Pooled investments:					
U. S. Government securities	32,984	5.0	31,391	4.5	1,386
Corporate bonds	206,043	31.0	194,703	27.7	6,333
Common stocks	425,496	64.0	475,851	67.8	15,759
	664,523	100.0	701,945	100.0	23,478
Less temporary investment of current fund cash	(5,728)				(254)
	658,795				23,224
Other investments:					
U. S. Government securities	27,938				1,128
Other bonds	15,029				748
Preferred stocks	3,448				168
Common stocks	58,886				65,814
Real estate	16,069				
	121,370				67,858
Total investments of other endowment and unrestricted funds	$ 780,165				91,082
Total					178,932
Custodian's fees charged thereto					(3,069)
Investment income distributed to invested funds					175,863
Plant investments:					
Federal agency and corporate bonds ..	$ 50,000				3,241
Current investments:					
U. S. Treasury bills, due February 2, 1967	$ 58,219				3,106
Temporary investment in pooled securities	$ 5,728				254
					3,360
Total investment income					$182,464

THE EFFECT OF TRYPSIN ON REGENERATION INHIBITORS IN TUBULARIA[1]

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Regeneration can occur at all levels along the stem of the marine hydroid, *Tubularia.* In cut pieces of stem the end that was nearest the hydranth usually regenerates a new hydranth, whereas the other extremity of the piece usually regenerates a new proximal end.

The region formed first during reconstitution is the hydranth. Once it is formed it can exert an influence on the other regions of the stem fragment. The hydranth is referred to as the dominant region because it controls the morphogenetic processes of the other regions in its field. (The term *field* implies the area in which some agent is at work in a co-ordinated way establishing an equilibrium within the area [Child, 1941].). In the intact animal the hydranth has the ability to prevent differentiation of another hydranth in the field it dominates. When the hydranth is removed from a stem fragment, regeneration occurs.

Rose and Rose (1941) found that there must be enough cut surface of the stem exposed to the sea water environment to allow escape of an inhibiting substance. They collected "inhibitor water" from aerated sea water containing many cut hydranths. This water was capable of inhibiting regeneration of newly cut stems. Heating destroyed the inhibitory effect of the water.

Fulton (1959) collected inhibitor water in low concentrations of either streptomycin or penicillin. Since this water did not affect regenerating stems, he suggested that the activity of inhibitor water might be due to bacteria or their metabolic products. Since Fulton's re-examination of the inhibitor, Tweedell (1958b) has produced a bacteria-free inhibitor water. Rose (1955, 1957, 1961, 1963) has been able to demonstrate inhibitory effects with grafts of living tissue when bacterial action was not a factor. Grafts of hydranth primordia, correctly oriented in a distal position, suppress the development of homologous portions of the host. Tweedell (1958a, 1962) and Powers (1961) have shown that tissue extracts from adult hydranths can inhibit reconstitution of stems. Finally, Rose (1963) has described an inhibiting substance obtained from primordia that can be moved electrophoretically. The present experiments were designed to study further this substance that can be extracted from regenerating *Tubularia* and can inhibit regeneration.

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METHODS

Tubularia crocea was used as an experimental animal because of its rapid rate of regeneration and its easy availability near the Marine Biological Laboratory at Woods Hole, Massachusetts, where this research was done.

Colonies of *Tubularia* were collected freshly for each experiment from the Cape Cod Canal. The colonies were kept cool during transport by immersing plastic bags filled with ice cubes in their container. The animals were brought into the laboratory as soon as possible and placed in running aerated sea water.

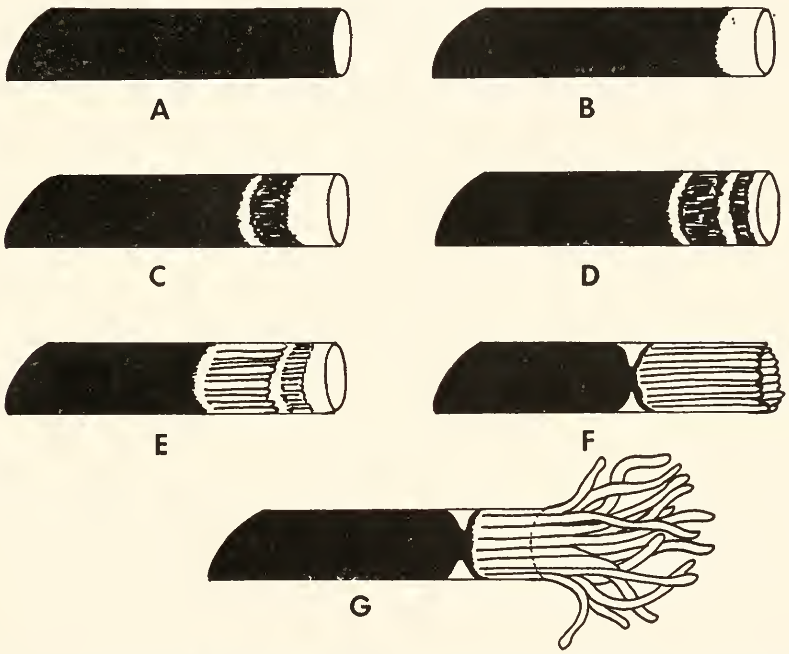

FIGURE 1. Stages in *Tubularia* regeneration. A, inactive; B, activated; C, one-band; D, two-band; E, two-band striated; F, constricted; G, emerging.

Stems were individually cut from the colony. The distal end was severed 5 mm. from the hydranth perpendicular to the vertical axis of the stem; the proximal cut was made obliquely to the vertical axis of the stem 6 mm. from the distal end. The stems were placed in glass fingerbowls of filtered pasteurized sea water containing chloromycetin (100 mg./l.) to control bacterial growth and were allowed to develop under a flow (300 ml./min.) of filtered sea water. Cheesecloth covers prevented the stems from flowing out of the fingerbowls.

The stems were allowed to regenerate approximately 15 hours. Stems that were in the two-band striated stage (Fig. 1E) were individually selected. Thirty to 35 future hydranth regions were cut from these stems. These primordia were homogenized in 1 ml. of pasteurized filtered sea water.

The homogenate was centrifuged at 5500 *g* for 5 minutes. A portion of the supernatant was pipetted into a "well" (8 × 2 × 4 mm.) which had been cut in electrophoretic starch. In addition to primordial homogenate, 0.05 ml. of each of the following four other solutions was electrophoresed: (1) 0.5 ml. of primordial supernatant + 0.08% trypsin in sea water, (2) 0.5 ml. of sea water + 0.08% trypsin in sea water, (3) 0.5 ml. of sea water + 0.30% trypsin in sea water, (4) sea water.

The starch was made with borate buffer (pH 8.6) which had been diluted 10 times with distilled water. Platinum electrodes transmitted the current from a Vokam Power Supply (type 2541) through the undiluted buffer in the electrode wells to filter paper wicks in contact with the starch. The gel was covered with a thin coating of paraffin oil to prevent drying and cracking of the blocks. Ice cubes were used to maintain the temperature at 15–16° C. Electrophoresis was carried out for three hours at 150 volts with a milliamperage of 5. The remaining supernatant was pipetted from the well when electrophoresis was terminated. Duplicate blocks were simultaneously electrophoresed. The duplicates were subsequently stained with amido black and then destained to estimate the mobility of proteins through the gel. These blocks indicated that an area 5 mm. from the edge of the well might contain a relatively large amount of material that had been moved through the starch gel.

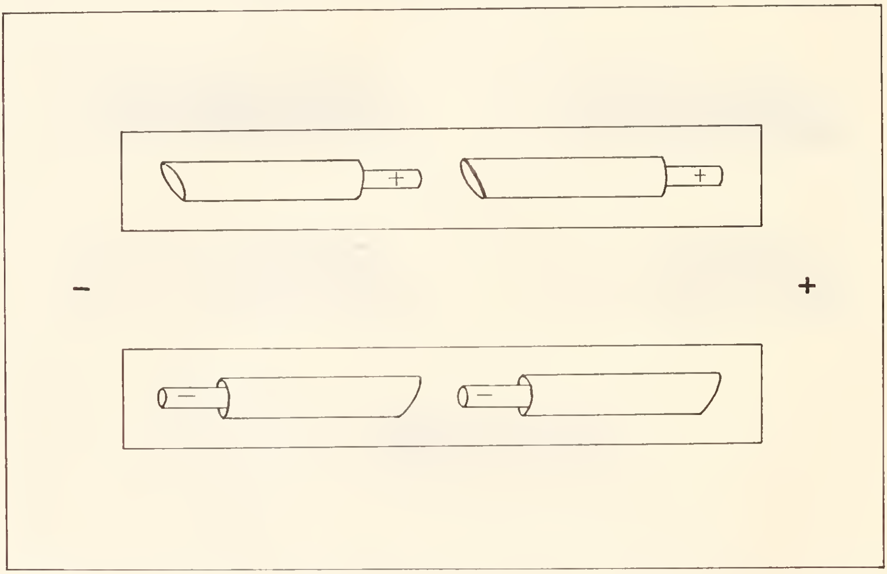

Figure 2. *Tubularia* stems containing starch cores with charged material oriented in electric field.

Glass coring tubes whose bores were 0.3–0.4 mm. were used to make plugs from the starch 5 mm. on both sides of the well. The starch gel core was forced out of the tube by applying heat to the larger opposite closed end of the coring tube. Starch plugs were made from the previously described electrophoresed blocks. They were inserted into the distal ends of *Tubularia* stems individually cut from a single colony. The stems selected had all reached the one-band stage in regeneration (Fig. 1C). The plugs were placed with approximately one-third of their total length within the coelenteron. Any stem which had been physically damaged to the slightest extent during plugging was not used for experimental purposes.

The stems were placed in V-shaped grooves cut in a tray of 2% agar gel made with filtered pasteurized sea water containing chloromycetin (100 mg./l.). They were oriented so that any charged material in the plugs would be drawn into the animal when a current was applied (Fig. 2). (Pilot experiments had revealed that the plugged stems had to be placed in an electric current for the primordial extract gel to inhibit the rate of regeneration.)

A series of experiments was conducted to determine the current to which *Tubularia* could be subjected for 8 hours and still undergo typical regeneration. A current of 1 m.a./5.7 mm.^{2} caused slowing of the rate of regeneration and some reversible damage to the stems. A current of 1 m.a./6.0 mm.^{2} slightly retarded the rate of reconstitution. The current used in these experiments (1 m.a./68 mm.^{2}) did not visibly damage the stems and allowed typical regeneration. There was no evidence of the reversal of polarity which has been reported when higher currents were applied over longer periods (Barth, 1934; Levin, 1961).

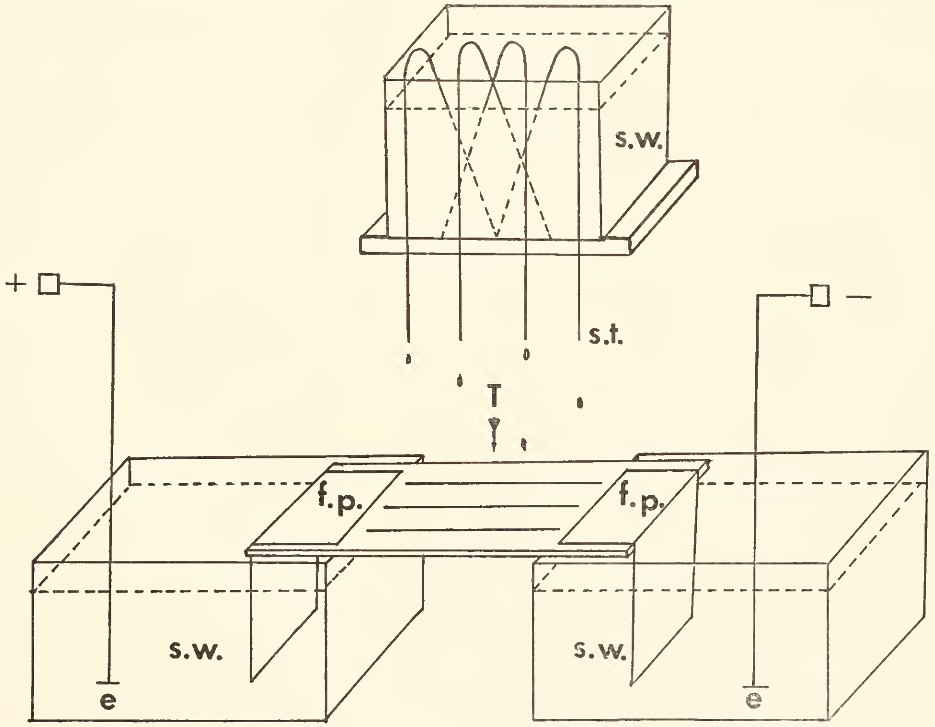

FIGURE 3. *Tubularia* in agar tray exposed to an electric field (6 volts; 10 milliamperes) for 8 hours. *e,* platinum electrode; *s.w.,* filtered pasteurized sea water containing chloromycetin; *f.p.,* filter paper wicks; T, *Tubularia* stems in grooves cut in tray of agar; *s.t.,* siphon tube.

A plastic tray (173 × 85 × 8 mm.) containing plugged *Tubularia* and a group of unplugged stems was placed in an electric current of 10 milliamperes and a voltage of 6 for eight hours (Fig. 3). The temperature was maintained at 15–16° C. during the entire eight hours. The stems were under a constant flow (100 ml./hr.) of filtered pasteurized sea water containing chloromycetin (100 mg./l.) This

TABLE I

*Experiment 1**

Number of Tubularia in each regeneration stage eight hours after plugging

Type of electrophoresed material contained in the plugs	Inactive	Activated	1-Band	2-Band	2-Band striated	Constricted	Emerging
(+) Primordia	5	1	1	0	1	2	0
(−) Primordia	5	1	1	1	0	0	0
(+) Primordia & 0.08% trypsin	1	0	0	0	1	6	0
(−) Primordia & 0.08% trypsin	1	0	0	0	2	5	0
(+) Sea water & 0.08% trypsin	1	1	0	1	0	5	0
(−) Sea water & 0.08% trypsin	2	1	0	1	1	3	0
(+) Sea water & 0.30% trypsin	1	2	0	0	1	3	1
(−) Sea water & 0.30% trypsin	3	0	0	1	0	4	0
(+) Sea water	2	0	0	1	2	2	1
(−) Sea water	1	0	0	0	0	5	1
Unplugged controls	1	0	0	1	0	6	0

* This experiment was repeated three more times with similar results.

flow was delivered by multiple overhead capillary siphon tubes. The electrode wells were filled with sea water.

At the end of eight hours the stems were removed from the current and classified into one of seven regenerative stages (Fig. 1): inactive (newly cut), activated, one-band, two-band, two-band striated, constricted, and emerging. Davidson and Berrill (1948) and Steinberg (1954) described detailed characteristics of the stages of *Tubularia* regeneration. Over 325 stems were studied in these experiments. The stems were placed in stender dishes at 15° C. for further observations.

RESULTS

The regeneration stages reached by stems eight hours after plugging in the first experiment are indicated in Table I. These experiments were repeated four

TABLE II

Percentage of stems at two-band or a more advanced stage of regeneration eight hours after plugging. (Means of four experiments)

Type of electrophoresed material contained in the plugs	Number of stems	Mean percentage
(+) Primordia	33	49
(−) Primordia	36	32
(+) Primordia & 0.08% trypsin	31	93
(−) Primordia & 0.08% trypsin	32	84
(+) Sea water & 0.08% trypsin	32	75
(−) Sea water & 0.08% trypsin	32	62
(+) Sea water & 0.30% trypsin	24	62
(−) Sea water & 0.30% trypsin	22	68
(+) Sea water	28	71
(−) Sea water	28	85
Unplugged controls	31	83
Total	329	

times with similar results. The time involved in making the plugs and inserting them in the stems limited the number of stems involved in the experiment. Because this phase of the investigation was conducted over a five-week period and the regenerative rates of controls varied from colony to colony, it was not valid to designate stage-by-stage summation of all four experiments in a single chart. However, Table II indicates the mean percentage of stems in all four experiments that had reconstituted to a more advanced stage than the one-band stage they exhibited when the cores were inserted.

Tubularia plugged with gel from electrophoresed sea water regenerated at a rate comparable to those which were not plugged with any type of gel. The animals that received gel from electrophoresed homogenates of primordia regenerated at approximately half the rate of the controls. When the primordial material was combined with 0.08% trypsin, the stems developed at the same rate as the controls. The other two groups plugged with sea water plus trypsin starch gel indicated that trypsin did not increase the rate of regeneration; in fact, it somewhat retarded the rate of regeneration.

From these results it was concluded that a substance (or substances) from the supernatant of primordial tissue homogenate and separated by starch gel electrophoresis is capable of inhibiting the regeneration rate of *Tubularia*. The effect of this substance may be removed by the addition of a small amount of trypsin, thus indicating a polypeptide or protein nature for this inhibiting factor.

Discussion

In these experiments the factors which are known to influence the regenerative process have been controlled. Any slight temperature variations (Moog, 1941) were simultaneously experienced by all stems in each experiment, and thus did not cause the differences observed in rate of reconstitution. A constant uniform flow of sea water was maintained in an effort to provide constant oxygen tension (Miller, 1937, 1939; Barth, 1938, 1940; Rose and Rose, 1941) and hydrogen ion concentration (Miller, 1939; Goldin, 1942). Chloromycetin was added to the environment to control bacterial growth (Tweedell, 1958b). The low currents (Barth, 1934; Levin, 1961) used did not cause a reversal of polarity, nor did they inhibit the rate of regeneration.

The substance (or substances), which is moveable in an electric field, can act as an inhibitor of regeneration. This factor does not totally inhibit reconstitution in the concentration employed, although it often caused the stems to regress temporarily to a less differentiated stage (Table I). When these stems were removed from the current they proceeded to differentiate in a typical manner. Subsequent observations revealed that the same percentage of the inhibited stems eventually emerged in the inhibited groups and in the control groups.

Plugs containing homogenate from two-band striated stems were tested for their effect on the earlier one-band stage. This method was used because preliminary experiments indicated that homogenates from primordia of early regeneration stages did not inhibit development in later stages. Steinberg (1954) and Tweedell (1958a) found that the inhibitor acts only during early stages of regeneration.

Some stems plugged with gel containing electrophoresed primordial supernatant continued to regenerate at a rate similar to that of the controls (Tables I and II). There are three principal factors that may have contributed to this observation: (1) The concentration of inhibiting substance in some plugs may not have been sufficient to cause an observable effect. (2) *Tubularia* stems exhibit individual variation in their rate of reconstitution; therefore, some stems that would have developed rapidly could have been inhibited, but still have regenerated at the same rate as slower regenerating control stems. (3) There seems to be a critical period for using inhibitor substance obtained from one development stage on a less advanced stage. Some stems may have progressed beyond the period when the inhibitor substance could exert its optimal action.

Inhibitory material from primordia was found on both sides of the well. This could be explained by the presence of two similar, but differently charged inhibitors. It is well known that proteins exist in nature only as part of more complex systems. Indeed, even purified proteins may be formed by the union of several simpler components. Another possibility is that the inhibitor is a substance that is altered in some way so that it is fractionated into two active oppositely charged components.

A group of pilot experiments which were performed at pH 4.1 using acetate buffer indicated that the substance (or substances) was positively charged at this pH since no inhibitory material could be detected migrating toward the positive pole. More experiments at different pH's using techniques of finer separation will be needed to elucidate the chemical properties of the inhibitor.

Rose (1963) discussed the polarized control of differentiation during regeneration. He emphasized the hypothesis that (p. 490) "bioelectric fields determine the direction of flow of specific inhibitors, thus changing a totipotent system into a differentiated one."

The findings in these experiments lend support to Rose's hypothesis: *viz.* they confirm that there is an inhibitory agent (or agents) of a polypeptide or protein nature that can be moved in an electric field.

SUMMARY

1. Supernatant of homogenate obtained from regenerating *Tubularia* stems was electrophoresed through starch gel.

2. Starch cores from the gel were partially inserted into the coelenterons of stems in an earlier stage of reconstitution.

3. These stems were then oriented in an electric current so that any charged material in the cores would migrate toward the stems.

4. The electrophoresed supernatant from primordial homogenate retarded the rate of reconstitution.

5. The addition of a small amount of trypsin prior to electrophoresis removed the regeneration-inhibiting effect of the supernatant, thus indicating a polypeptide or protein nature for the inhibiting substance(s).

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CHEMORECEPTION IN THE MUD SNAIL, NASSARIUS OBSOLETUS. I. PROPERTIES OF STIMULATORY SUBSTANCES EXTRACTED FROM SHRIMP[1]

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Chemoreception is believed to play a role in many aspects of gastropod behavior (Kohn, 1961). Studies have suggested that chemoreceptive mediation is involved with such diverse phenomena as carrion location by scavengers (Copeland, 1918), metamorphosis (Scheltema, 1961), plant recognition by herbivores (Frings and Frings, 1965), predator avoidance (Bullock, 1953), prey recognition by predators (Blake, 1960), sexual differentiation (Coe, 1953), and others. The compilation of a sizable literature on the ecological significance of chemoreception to aquatic gastropods and aquatic invertebrates in general (for review, see Hodgson, 1955) is contrasted with the paucity of available information on the molecular aspects of such problems. A satisfactory understanding of aquatic chemoreceptive phenomena can be attained only after insight is gained into the molecular nature of the compounds which are involved.

Previous studies on chemoreception in marine gastropods have been limited in their scope by the fact that the animals selected for study and/or the techniques employed have denied investigators a chance to progress much beyond the "observation of response" stage of investigation. However, in the present study the mud snail, *Nassarius obsoletus,* is shown to be an extremely suitable animal for studies of chemoreception. Dimon (1905) noted that this marine gastropod possessed a chemical sense and could detect the "odor or taste" of substances which diffused from dead animals. Submerged individuals were observed by Copeland (1918) to respond to extracts of fish by extending their proboscides. Copeland referred to this response as the proboscis reaction. Prior to the present study no attempt has been made to identify the substances which are stimulatory to this snail.

The report which follows has a twofold purpose: (1) to describe a procedure for studying a chemically mediated response in *N. obsoletus;* and (2) to provide the results of experiments which characterized the response-inducing substances extracted from shrimp.

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[1] This paper is based on a portion of a dissertation submitted to the Graduate School of Arts and Sciences of Duke University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Zoology. These studies were conducted at the Duke University Marine Laboratory, Beaufort, N. C., and were supported by a N.I.H. Physiology Training Grant and by a Bureau of Commercial Fisheries Graduate Education Grant. The author is grateful to Dr. K. M. Wilbur, Department of Zoology, Duke University, for providing frequent counsel during the course of this research.

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METHODS

Maintenance of animals

Specimens of *Nassarius obsoletus* were collected near the Duke University Marine Laboratory, Beaufort, N. C. Prior to use, freshly collected snails were kept under running sea water in the laboratory for 12 to 14 hours for washing and egestion of intestinal contents. For further washing and a period of adjustment to room temperature, groups of approximately 200 snails were transferred to 8-liter glass aquaria filled with freshly pumped and aerated sea water. After the water reached room temperature (21–26° C.) it was replaced several times over a 24-hour period with water at the same temperature. Groups of 45 snails each were then transferred to 8-liter aquaria, each containing approximately 4 liters of filtered and continuously aerated sea water which was changed after three days. No tests were performed with snails which had been in the laboratory less than 48 hours. Snails were used no longer than 8 days after capture. Snails were seldom used for more than one test and never less than 24 hours after a previous test.

Snails with heavily eroded or encrusted shells or with obvious physical imperfections were not used. Between mid-December and early May only males were used in order to avoid variables resulting from copulation and capsule deposition in mixed populations. At other times mixed populations were used.

In order to keep organic contamination to a minimum, sea water for maintaining and testing washed snails was obtained from Beaufort Inlet at high tide. Water was collected in glass carboys, filtered, stored at 4° C., and warmed to room temperature as needed. The sea water in which snails were tested was always collected at the same time as the water in which these same snails were maintained. The salinity range was 29–35%.

Bioassay procedures

Bioassays were performed in an illuminated observation cubicle which was provided with a mirror to permit observation of the behavior of snails moving away from the observer (Fig. 1). Solutions were bioassayed in 10-cm. petri dishes. A solution volume of 40 ml., sufficient to cover the siphonal canal, was used. Bioassays were standardized as follows:

(1) Ten snails (comprising one test group) were transferred from an aquarium into a fingerbowl of sea water dipped from the aquarium. The snails were placed in the observation cubicle for 10–15 minutes prior to beginning the bioassays.

(2) Individuals were transferred with stainless steel forceps into the solution which was to be bioassayed. The snails were tested one at a time. A one-minute testing period was allowed for displaying the proboscis search reaction (description in Results).

(3) A single solution was used for one test group. Forceps used in the transfer of snails were rinsed thoroughly in distilled water after each transfer. Water in the containers used for rinsing snails or forceps (see Fig. 1) was changed after each series of bioassays involving a test group.

Preparation of shrimp extracts

The shrimp (*Penaeus duorarum*) used as the source of tissue for the studies of response-inducing substances were transported to the laboratory alive, placed into

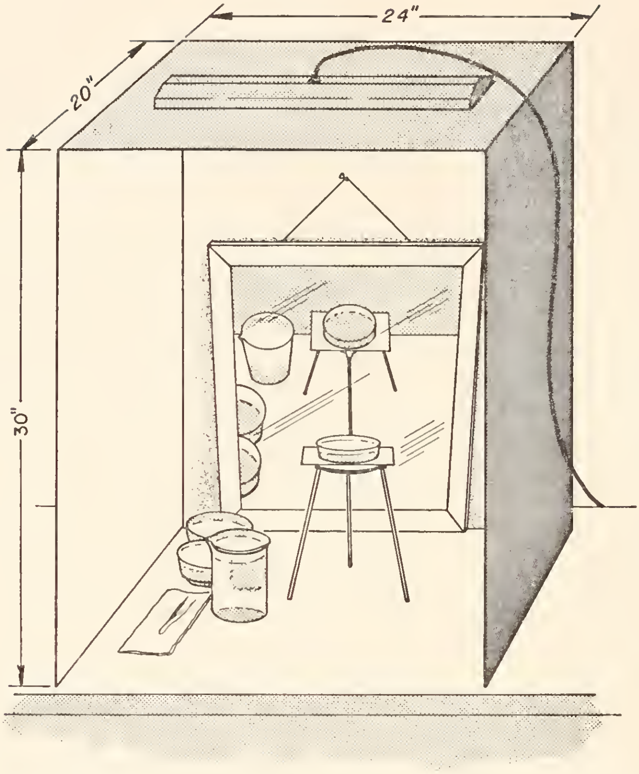

FIGURE 1. The observation cubicle. The cubicle was constructed from half-inch plywood and had the following dimensions: height = 30 in.; width = 24 in., depth = 20 in. A slot cut from the top provided for a framed 15-watt, Cool White General Electric fluorescent light. The mirror (39 × 49 cm.) hanging diagonally in the rear of the cubicle permitted the observation of the behavior of snails moving away from the observer. The following items were located within the cubicle: (1) forceps used for transferring snails; (2) ring stand to support test solution; (3) fingerbowl of water to rinse snails after they were used; (4) fingerbowl of sea water to hold snails after they were used; and (5) beaker of distilled water for rinsing forceps.

plastic bags, and stored in a deep freeze. For all extracts the abdomen was used without appendages and exoskeleton. Extracts were prepared in two ways:

(1) Aqueous extracts (10:1, v/w) were prepared by homogenization of shrimp for 5 minutes in a Waring Blendor. The homogenates were filtered with suction through Whatman No. 5 paper, refiltered through a Millipore filter (0.45-μ pore size), and kept in ice.

(2) Chloroform:methanol (2:1) extracts were prepared according to the procedure of Folch, Lees and Sloane Stanley (1957). After addition of 0.2 volume of water to the filtered homogenate, the preparation was stirred for several minutes and phase separation was accelerated by centrifugation at 400 g for 20 minutes. The upper aqueous phase was removed, evaporated to dryness in an oven at 50° C., suspended in water (1 ml./1 g. initial shrimp weight), filtered through a Millipore

filter, and kept in ice. Upper phase material was used extensively and will be designated shrimp extract. Lower phase material was not used except when specified.

Shrimp was selected as the source of tissue for these studies because of its availability and because only a minimum amount of manipulation is necessary to prepare it for extraction.

Procedures for studying the properties of stimulatory substances extracted from shrimp

The following properties of the stimulatory substances extracted from shrimp were studied: stability to heat, solubility, volatility, dialyzability, and stability to acid and ammonia treatment. The molecular-charge properties of the stimulatory substances were studied by means of a series of fractionations of extracts on ion exchange columns. In each experiment, a portion of an extract was treated (or fractionated) in the manner described below. Tests were carried out which compared the response-inducing capacity of the treated (or fractionated) extract with the response-inducing capacity of the untreated (or total) extract.

Whenever necessary, solutions were evaporated to dryness by rotary evaporation *in vacuo.* All preparations were kept in ice and tested as soon after preparation as possible.

Heat treatment. Sealed aliquots of aqueous shrimp extract were suspended in a boiling water bath for periods of 1 and 12 hours. The preparations were filtered and tested.

Solubility studies. Prior to deciding upon an extraction procedure to be used for further studies, it was necessary to get an approximation of the relative solubility of the stimulatory substances in a series of solvents of differing polarity. Ten-ml. aliquots of aqueous shrimp extract were evaporated to dryness and to each residue were added 10 ml. of one of the following solvents: water, methanol, ethanol, chloroform : methanol (2 : 1), chloroform : methanol : water (3 : 48 : 47), acetone, ether. The containers were stoppered, placed on a shaker for 30 minutes, filtered, and evaporated to dryness. Each residue was dissolved in 10 ml. of water and tested.

Ultrafiltration. An aliquot of shrimp extract was ultrafiltered at 0–1° C. through 6-mm. Visking tubing according to the procedure of Smith (1960, pp. 60–62). The ultrafiltrate was tested.

Acid hydrolysis. A 1-ml. aliquot of shrimp extract was hydrolyzed in 6 N HCl (20 hours, 100° C.). The hydrolysate was evaporated to dryness and the residue was dissolved in 1 ml. of water, neutralized with NaOH, and tested.

Ion exchange separations. The neutral compounds in shrimp extract were collected by passing a 2-ml. portion of extract through a 1.2 × 12-cm. column of Bio-Rad AG 501-X8 (mixed bed resin, Analytical Grade, 20–50 mesh, Calbiochem). Ten bed volumes of water effluent were collected at a flow rate of 0.5 ml. per minute. The effluent containing neutral compounds was evaporated to dryness and the residue was dissolved in 2 ml. of water for testing.

The neutral, acidic, amphoteric, and weakly basic compounds in shrimp extract were separated from the strongly basic compounds by passing a 3-ml. portion of extract through a 1 × 15 cm. column of Rexyn 102 (Chromatographic Grade, 200 mesh, Fisher). The procedure of Awapara *et al.* (1960) for the separation of amino acids from amines was used with the following modifications: (1) 10 bed volumes of water effluent were collected; (2) elution was carried out with 10 bed volumes of 1 N HCl. The effluent (containing neutral, acidic, amphoteric, and weakly basic substances) and the eluate (containing strongly basic substances) were collected at a flow rate of 0.5 ml. per minute. Each was evaporated to dryness and the residue was dissolved in 3 ml. of water for testing.

A 1 × 11 cm. column of Dowex 50W-X8 (Analyzed Reagent, 200–400 mesh, Baker) was used according to the procedure of Smith (1960, p. 59) to separate the components of shrimp extract into 3 fractions: (1) a water effluent containing primarily neutral and acidic compounds; (2) a 2 N-NH_3 eluate containing amphoteric and weakly basic compounds; and (3) a 10 N-NH_3 eluate containing strongly basic compounds. One ml. of extract was added to the column and 10 bed volumes of each of the above fractions were collected at a flow rate of 0.5 ml. per minute. Each fraction was evaporated to dryness and the residues were dissolved in one ml. of water for testing. In a separate experiment, 2.5 ml. of extract were added to a 1 × 15 cm. column of Dowex 50W-X8. Ten bed volumes each of water effluent and 2N-NH_3 eluate were collected, evaporated to dryness, and the residues were dissolved in 2.5 ml. of water for testing.

Statistical treatment of data

Many of the data which follow were analyzed by Potency Probit Analysis employing the computer program of Daum and Givens (1963) for quantal (all-or-none response) bioassays. This program employs the maximum likelihood procedure of Finney (1962) and involves a regression analysis of the $\log_{10}$-dose *versus* probit response for a series of up to 5 simultaneous sets of data. The potency (ratio of equally effective doses) and its 95% confidence limits are computed for an "unknown"(s) with respect to a "standard." This analysis also provides calculation of an ED_{30} (effective dose for 30% of tested animals), ED_{50}, ED_{70}, and ED_{90} for each set of data which comprises a significant linear regression; these calculations permit the plotting of computed regression lines whenever it is desirable to present such material graphically. The computer program was provided by Mr. Kenneth Fischler, Biometrician, U. S. Fish and Wildlife Laboratory, Beaufort, N. C. The Potency Probit Analysis was carried out in the Duke University Digital Computing Laboratory.

RESULTS AND DISCUSSION

In the field *Nassarius obsoletus* quickly detected substances which leached from freshly killed crabs, fish, oysters and other animals. Submerged snails which were below the tide mark, where slight currents were sufficient to transport diffusing substances, were observed to move upcurrent directly to such material from distances of two or three feet. Such movements were accompanied by a horizontal waving of the siphon. Once a snail was within a few centimeters of fresh tissue, the food search was climaxed by a series of short and/or long extensions of the previously unexposed proboscis (Fig. 2). The initial contact of a snail with food

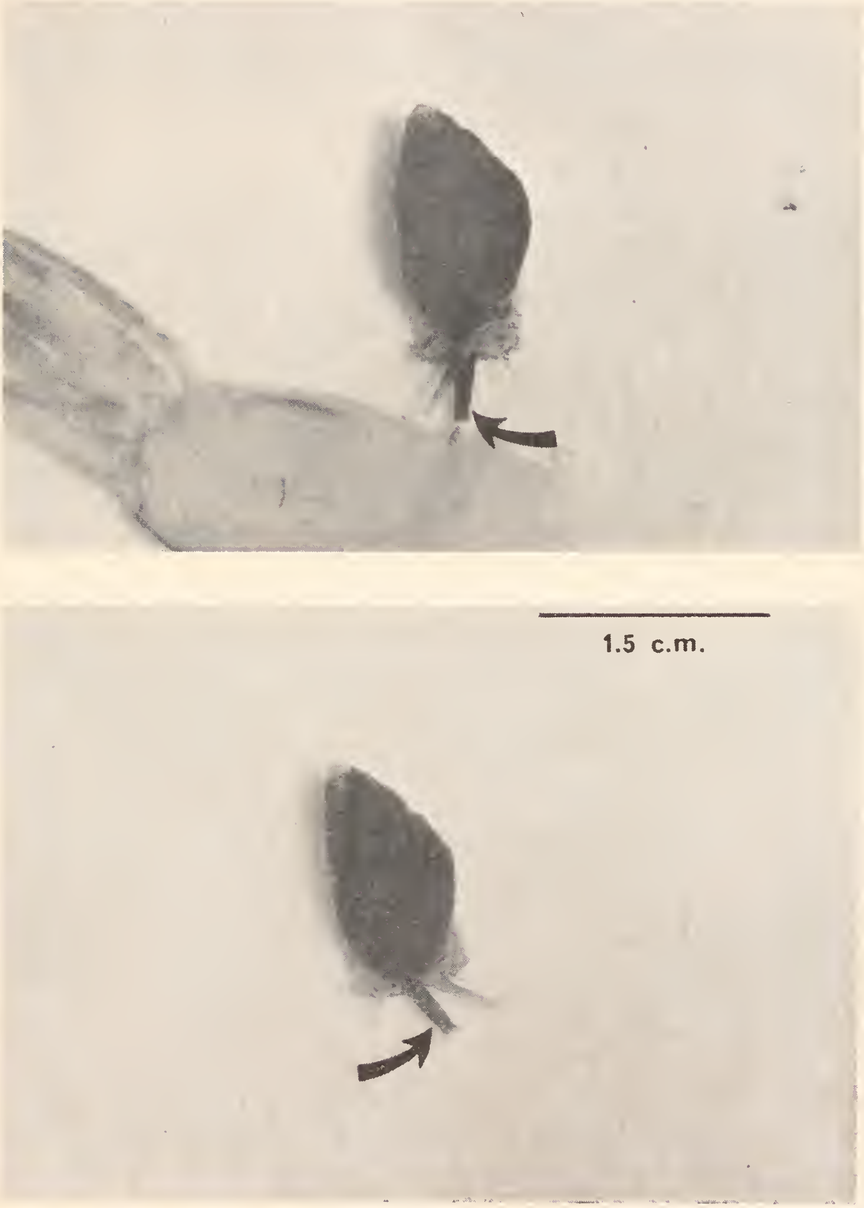

FIGURE 2. *N. obsoletus* responding to components released from fresh shrimp. The snail in upper photograph is submerged in sea water containing a portion of a shrimp (lower center). This snail is extending its proboscis (indicated by arrow) in response to substances diffusing from the shrimp. The snail in lower photograph is submerged in sea water which contained a portion of a shrimp prior to the introduction of the snail. This snail is extending its proboscis (indicated by arrow) in response to substances which were released from the shrimp.

was usually made by the proboscis; however, extensions of the proboscis (hereafter referred to as the proboscis search reaction [PSR]) always began before the snail contacted the food. In tidal pools two to four feet in diameter, the introduction of freshly killed oysters, fish or crabs resulted in accelerated locomotion and siphon waving as well as emergence from the mud of previously buried snails. Without the directional stimulus or gradient provided by a current, the movement of snails was frequently unrelated to the position of the food. Nevertheless, individuals which moved within a few centimeters of the food would begin the characteristic PSR and quickly locate the stimulus source. Early laboratory experiments revealed that the actual presence of food in sea water was not required to induce the PSR. The PSR was stimulated by the presence of sufficient concentrations of certain tissue substances which leached out after injury or death.

The distinctive feature of the PSR is a series of short (just beyond the anterior end of the siphonal canal) and/or long (siphon length) extensions of the proboscis. The reaction occurs within a few seconds. Preliminary observations on several hundred snails tested individually suggested that the response of an individual to a control or test solution during a 1-minute test period could be graded as follows:

0–3 extensions of the proboscis = No response
4 or more extensions of the proboscis = Satisfactory response.

These response criteria were used throughout. The initial expansion of the foot by a snail placed in a control or a test solution was sometimes accompanied by a short extension of the proboscis; also contact of a snail with the side of a testing dish sometimes resulted in one or two short extensions. In order to exclude such responses the grading system was established. The terms, stimulation, stimulatory solution, response, response-inducing solution, are references to the PSR or to a solution which induced the PSR.

Studies of the properties of stimulatory substances extracted from shrimp

Preliminary experiments revealed that the addition of small amounts of aqueous shrimp extracts to sea water yielded very stimulatory solutions. The following experiments were carried out to provide information on the properties of the stimulatory substances. In each of these experiments, tests of the untreated (or total) extract were conducted together with tests of the treated (or fractionated)

TABLE I

Responses of N. obsoletus to untreated and heat-treated shrimp extract

Extract concentration tested (μl./40 ml.)	Untreated extract		1 hr. heat-trtd. extract		12 hr. heat-trtd. extract	
	(1) No. snails tested	(2) % Response	(3) No. snails tested	(4) % Response	(5) No. snails tested	(6) % Response
0	10	10				
5	20	40	20	45	20	40
10	20	70	10	60	10	80
20	10	70	10	100	10	70

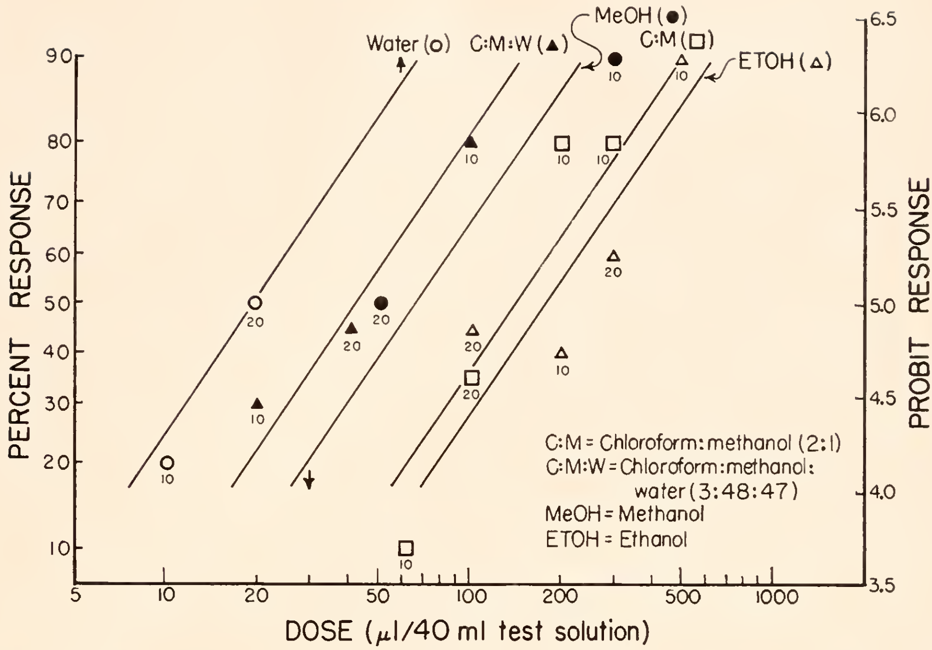

FIGURE 3. Responses of *N. obsoletus* to shrimp components soluble in water and organic solvents. The experimental dosage-response values are indicated by symbols; numbers next to symbols indicate numbers of snails tested. Linear regression lines were drawn from computed effective dose values obtained by Potency Probit Analysis. The upright arrow over the regression line for water-soluble substances indicates that a 100% response was obtained with the largest dose tested. The inverted arrow beneath the regression line for methanol-soluble substances indicates that no response (0%) was obtained with the smallest dose tested. The potencies (and 95% confidence limits) of the indicated preparations were computed with respect to the potency of the preparation containing water soluble substances:

	Upper lim.	0.867		Upper lim.	0.257
C : M : W	Potency	0.469	C : M	Potency	0.144
	Lower lim.	0.253		Lower lim.	0.079
	Upper lim.	0.577		Upper lim.	0.202
MeOH	Potency	0.307	ETOH	Potency	0.113
	Lower lim.	0.157		Lower lim.	0.065

extract. This measure insured that all groups of snails used in comparative tests were in the same physiological condition.

Effect of heat treatment. The results of bioassays of heat-treated and untreated shrimp extract are given in Table I. Heat treatment had no detectable effect upon the stimulatory capacity of the extract. This conclusion was based upon a comparison of the response percentages in columns 2, 4, and 6 of the Table.

Solubility studies. Figure 3 incorporates the experimental results of the solubility studies and the dosage-response regression lines obtained by a Potency Probit Analysis (see Materials). The figure shows the relative solubility of the stimulatory substances (from shrimp) in water, methanol, ethanol, chloroform:methanol, and chloroform:methanol:water. The stimulatory substances were somewhat more soluble in water than in the other solvents. However, with respect to solvents other than water, only the data from tests of substances soluble in chloroform: methanol:water and ethanol are significantly different. The figure emphasizes the existence of a relationship between the concentration of a stimulant and the percentage of snails responding to it. This relationship implied that different individuals possessed different thresholds and that the procedure of recording response as a function of dose over a range of concentrations provided a means of comparing solutions in terms of their stimulatory capacities. This procedure was used in the studies which follow. One of the shortcomings in using small numbers of animals is exemplified in the figure by the aberrant point obtained with ethanol-soluble material; a 40% response (10 animals tested) was obtained with a dose of 200 μl./40 ml. while a 45% response (20 animals tested) was obtained with a lesser dose of 100 μl./40 ml. Deviations such as this were compensated for by bioassaying solutions over a range of concentrations (minimum of three unless otherwise stated) and basing most evaluations upon a Potency Probit Analysis.

The stimulatory substances were only very slightly soluble in acetone and ether; data from these tests were not included in Figure 3. At concentrations of 5000 μl./40 ml., the substances soluble in the non-polar solvents (acetone and ether) induced but 2 responses (20%) each from single groups of snails. Since the water-soluble substances induced a 50% response at a concentration of only 20 μl./40 ml., the limited effectiveness of the substances soluble in the non-polar solvents implied that lipoidal and long-chain aliphatic substances were not the major response-inducers.

The extraction procedure of Folch *et al.* (1957) incorporated several features which made it a suitable technique for the acquisition of substances for further studies. After initial homogenization in chloroform:methanol (2:1), followed by the addition of 0.2 volume of water, a biphasic system results: an upper aqueous phase (chloroform:methanol:water, 3:48:47) and a lower lipoidal phase (chloroform:methanol:water, 86:14:1). The extract is essentially protein-free and after phase separation the upper phase is nearly lipid-free. The previous experiment showed that the solubility of the stimulatory substances in chloroform:methanol: water (3:48:47) was at least as great as the solubility of these substances in methanol and ethanol (see Fig. 3). An experiment was carried out to observe the distribution of the stimulatory substances in the biphasic system described above. The results revealed that the substances which were response-inducing at low concentrations were confined to the upper aqueous phase. The substances in the lipoidal phase were effective only at concentrations which were approximately tenfold greater. Further references to shrimp extracts signify the utilization of the upper phases from extracts prepared according to the procedure of Folch *et al.*

Tests of volatile components. The fact that previous extracts were evaporated to dryness prior to use suggested that volatile components were not the ones stimulating the PSR. Nevertheless the findings of Brown (1961) and Kleerekoper and Mogensen (1963), concerning the chemoreceptive importance of volatile amines to certain aquatic animals, prompted experiments to collect and bioassay volatile components. However, the response-inducers extracted from shrimp were nonvolatile substances. Volatile amines collected from shrimp by steam distillation were not response-inducing. Equally ineffective were volatile substances which

TABLE II

Responses of N. obsoletus to total and ultrafiltered shrimp extract

Concentration tested (µl./40 ml.)	Untreated extract		Ultrafiltrate of extract	
	No. snails tested	% Response	No. snails tested	% Response
0	10	0		
10	10	30	10	30
20	20	45	20	55
40	10	70	10	80

Potency* (and 95% confid. lims.) of ultrafiltrate

Upper limit	3.95
Potency	1.27
Lower limit	0.60

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* The potency of the ultrafiltrate was computed with respect to the potency of the untreated extract.

were drawn from shrimp by negative pressure and trapped in cold sea water for testing.

Effect of ultrafiltration. The results of bioassays of shrimp extract and an ultrafiltrate of the same are given in Table II. The ultrafiltrate was as effective as the total extract. The results indicated that the principal response-inducers were substances of low molecular weight.

Effects of acid and alkaline hydrolysis. Shrimp extracts contained an acid-labile component(s) which contributed to the stimulatory capacity. Extract hydrolyzed in 6 N HCl was markedly less effective than untreated extract (Table III). Another similar experiment gave similar results. In a separate experiment, portions of shrimp extract were treated with 2 N HCl or 2 N NH_3 (1 hour at

TABLE III

Responses of N. obsoletus to untreated and acid-hydrolyzed shrimp extract

Concentration tested (µl./40 ml.)	Untreated extract		Acid-hydrolyzed extract	
	No. snails tested	% Response	No. snails tested	% Response
0	10	0	—	—
5	10	10	—	—
10	20	50	—	—
20	20	65	10	10
40	—	—	20	25
80	—	—	20	65

Potency* (and 95% confid. lims.) of acid-hydrolyzed extract

Upper limit	0.312
Potency	0.194
Lower limit	0.108

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* The potency of the acid-hydrolyzed extract was computed with respect to the potency of the untreated extract.

100° C.). The acid treatment resulted again in a decrease in the stimulatory capacity, while treatment with NH_3 did not.

Effect of oxidation. Although no single experiment was designed to observe the effect of oxidation, other treatments (*i.e.*, evaporation of extract to dryness in an oven at 50° C. and retention of activity after 12 hours in boiling water bath) implied that readily oxidizable substances were not among the principal contributors to the stimulatory capacity of shrimp extracts.

Ion exchange separations. The neutral compounds in shrimp extract were collected by passing a portion of the extract through a column of mixed bed resin. Bioassays revealed that the neutral compounds were essentially non-stimulatory even at high concentrations (concentrations proportional to 250 and 500 μl. of extract per 40 ml.). The total extract itself induced a response of 55% at a concentration of only 5 μl./40 ml. These results verified those obtained from an earlier similar separation which was bioassayed over a smaller range of concentrations.

TABLE IV

Responses of N. obsoletus to shrimp extract and to extract components collected in water effluent and acid eluate from a column of Rexyn 102

Material tested	Concentration tested (μl./40 ml.)	No. snails tested	% Response	Potency* (95% confid. lims.)
Total shrimp extract	0	10	0	
	2	20	35	
	5	20	55	
	10	20	60	
	20	20	75	
Water effluent from Rexyn 102 column	5	20	50	Upper lim. 3.45
	10	20	55	Potency 0.68
	20	20	65	Lower lim. 0.14
Acid eluate from Rexyn 102 column	20	10	0	
	40	10	0	
	100	10	0	
	250	10	10	
Water effluent plus acid eluate	5	10	50	
	10	10	60	

* Potency (and 95% confidence limits) of water effluent was computed with respect to potency of the total shrimp extract.

Shrimp extract was passed through a column of Rexyn 102 to separate the neutral, acidic, amphoteric, and weakly basic compounds from the strongly basic compounds. Results of the tests are given in Table IV. The compounds in the water effluent (neutral, acidic, amphoteric, and weakly basic compounds) possessed a stimulatory capacity comparable to that of the total extract. The acid eluate (containing strongly basic compounds) was very ineffective. Combinations of the effluent and eluate were not detectably more effective than the effluent alone. These results verified those obtained from an earlier, similar separation which was bioassayed over a smaller range of concentrations.

The previous experiment suggested that the principal contributors to the response-inducing capacity of shrimp extracts were included with the neutral, acidic, amphoteric, and weakly basic compounds. Shrimp extract was passed through a column of Dowex 50W-X8 to separate the neutral and acidic compounds (collected in the water effluent) from the amphoteric and weakly basic compounds (collected in the 2 N-NH_3 eluate). Strongly basic compounds were collected by a terminal elution of the column with 10 N NH_3. Results of the tests are given in Table V. The components present in the 2 N-NH_3 eluate were markedly more effective than the components present in either the water effluent or the 10 N-NH_3 eluate. However, components in the 2 N-NH_3 eluate were somewhat less effective than the total extract. The results were in accord with those obtained from an earlier, similar separation which was bioassayed over a smaller range of concentrations.

TABLE V

Responses of N. obsoletus to shrimp extract and to extract components present in water effluent, 2 N-NH_3 eluate, and 10 N-NH_3 eluate from a column of Dowex 50W-X8

Material tested	Concentration tested (µl./40 ml.)	No. snails tested	% Response	Potency* (95% confid. lims.)
Total shrimp extract	0	20	10	
	10	20	50	
	20	20	65	
	40	20	70	
Water effluent from Dowex 50 column	20	10	20	
	40	10	20	
	100	10	10	
2 N-NH_3 eluate from Dowex 50 column	10	20	30	Upper lim. 0.946
	20	30	43	Potency 0.341
	40	10	60	Lower lim. 0.011
	100	10	70	
10 N-NH_3 eluate from Dowex 50 column	20	10	0	
	100	20	15	

* Potency (and 95% confidence limits) of 2N-NH_3 eluate was computed with respect to potency of the total shrimp extract.

The finding that the extract components in the 2 N-NH_3 eluate from the Dowex 50 column were less effective than the total extract suggested that one of the other fractions collected from the column contained an important component(s). The Rexyn 102 separation suggested that strongly basic compounds did not contribute to the extract's stimulatory capacity (see Table IV). This made it seem likely that the water effluent from the Dowex 50 column (containing neutral and acidic compounds) contained the important component(s). Another separation of shrimp extract on a column of Dowex 50W-X8 was carried out and the water effluent and

2 N-NH_3 eluate were tested individually and in combination (Fig. 4). The results revealed that the potency of the total extract was recovered when acidic and neutral compounds in the water effluent were combined with the amphoteric and weakly basic compounds in the NH_3 eluate. A Potency Probit Analysis showed that there was a significant difference between the stimulatory capacity of the total extract and the NH_3 eluate but that there was no significant difference in the effectiveness of the total extract and the combination of effluent and NH_3 eluate. As in previous, similar separations the water effluent alone did not induce many responses at the lower concentrations; however, this material contained a strong response-inducing component(s) which became evident at a concentration of 400 µl./40 ml. (65% response).

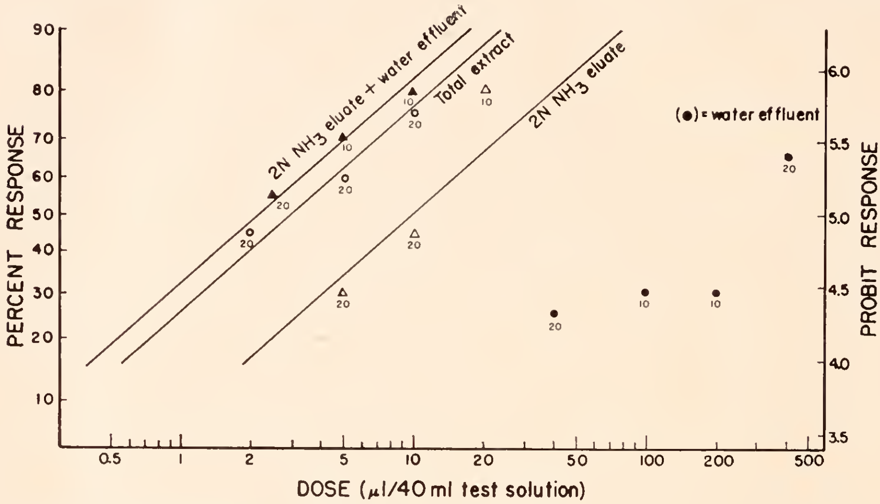

FIGURE 4. Responses of *N. obsoletus* to shrimp extract and to extract components collected in the water effluent and 2 N-NH_3 eluate from a column of Dowex 50W-X8. Linear regression lines were drawn from effective dose values obtained by a Potency Probit Analysis. The experimental dosage-response values are indicated by symbols; numbers next to symbols indicate numbers of snails tested. The following potencies were computed with respect to the potency of the total shrimp extract:

	Upper lim.	0.649		Upper lim.	4.623
2 N-NH_3 eluate	Potency	0.287	2 N-NH_3 eluate +	Potency	1.389
	Lower lim.	0.069	water effluent	Lower lim.	0.520

Studies of stimulatory components—A summary. The principal response-inducers in shrimp extracts possessed physical properties similar to those of the amino acids and certain other non-volatile, nitrogenous compounds of low molecular weight. These studies also indicated that proteins, labile esters, lipids, and volatile compounds were not important contributors to the response-inducing capacities of the extracts.

Ion exchange separations provided considerable insight into the nature of the principal response-inducers. Neutral compounds collected from a column of mixed bed resin were essentially ineffective even at 100 times the concentration necessary for the total extract to induce a response of greater than 50%. Separations of extracts on columns of cation exchange resins demonstrated that stimulation was attributable to amphoteric and/or weakly basic compounds which were augmented by some acidic and/or neutral compound(s).

Effects of small pH changes upon snail response

When necessary, treated (or fractionated) extracts were adjusted to pH 6–8 by a dropwise addition of NaOH. This measure ensured that the pH of each *test* solution was maintained within a considerable narrower range. To determine what effect slight changes in pH had upon snail response, a shrimp extract was tested in sea water which had been acidified by the addition of HCl. The results of these tests are given in Table VI. The snails were equally responsive over the specified pH range (5.8–8.0). In view of these findings that stimulation of the PSR was not effected by slight deviations in pH, test solutions were not buffered. Case (1964) employed electrophysiological techniques to study the sensitivity of crustacean chemoreceptors to amino acids, amines, and related compounds; he reported no change in the stimulatory efficiency of glycine or proline even over a larger pH range. Likewise, Levandowski and Hodgson (1965) found that pH changes in the range 5–8 had no detectable effects upon the responsiveness of lobster chemoreceptors to glutamic acid and certain amines.

TABLE VI

Responses of N. obsoletus to shrimp extract presented over a narrow range of pH

pH of test solution	Extract concentration tested (µl./40 ml.)	No. snails tested	% Response	Potency* (95% confid. lims.)
8.0 (unadjusted)	0	10	0	
	2	20	45	
	5	20	60	
	10	20	75	
6.9–7.4**	2	10	50	Upper lim. 3.46
	5	20	60	Potency 0.97
	10	10	70	Lower lim. 0.27
5.8–6.4***	0	10	0	Upper lim. 2.42
	2	20	40	Potency 0.84
	5	20	50	Lower lim. 0.24
	10	20	80	

* Potencies (and confidence limits) were computed with respect to the potency of the extract tested at pH 8.0.

** The pH of the sea water was 6.9 when tests were begun and 7.4 when tests terminated.

*** The pH of the sea water was 5.8 when tests were begun and 6.4 when tests terminated.

Merits of N. obsoletus as a test animal

N. obsoletus proved to be an excellent test animal for studies of chemoreception. This intertidal gastropod is easy to collect and is present in great numbers from the

Gulf of St. Lawrence to the northeast coast of Florida (Abbott, 1954, p. 240). The PSR is a stereotyped response which is convenient for measuring the stimulatory capacities of substances extracted from tissues. With the PSR as the criterion of response, it is possible not only to recognize response-inducing solutions but also to compare in a quantitative manner the response-inducing capacities of closely related solutions. The significance of the latter finding resides in the fact that this snail can be used as a tool for a thorough exploration of the factors (both chemical and physical) which contribute to and influence an integrated response in a marine gastropod. Further, *N. obsoletus* can be tested in small volumes of sea water and is easy to maintain in the laboratory; not a single snail died during the course of this work in which several thousand snails were used.

The development of a convenient bioassay procedure and the use of this procedure to characterize the response-inducing compounds in shrimp extracts have served as the basis for identifying and testing specific compounds, and combinations of compounds, which contribute to the capacity of these extracts to induce the PSR in *N. obsoletus*. The results of the latter experiments will be presented in a separate publication.

SUMMARY

1. The mud snail, *Nassarius obsoletus,* was shown to be an excellent test animal for studies of chemoreception. This snail responds to substances diffusing from dead animals by giving a series of extensions of the proboscis. With this response as the criterion, a bioassay procedure was developed for studying the properties of substances which contribute to the response-inducing capacities of shrimp extracts. *N. obsoletus* can be used in studies in which it is desirable to compare in a quantitative manner the response-inducing capacities of closely related solutions.

2. The principal response-inducing compounds in shrimp extracts were heat-stable, more soluble in polar than in non-polar solvents, non-volatile, of low molecular weight, alkali-stable, partially acid-labile, and resistant to oxidation.

3. Separations of shrimp extracts on ion exchange columns revealed that the principal response-inducers were amphoteric and/or weakly basic compounds which were augmented by some acidic and/or neutral compound(s).

4. Changes in pH within the range 5.8–8.0 had no detectable effect upon the responsiveness of *N. obsoletus* to a shrimp extract.

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CHEMORECEPTION IN THE MUD SNAIL, NASSARIUS OBSOLETUS. II. IDENTIFICATION OF STIMULATORY SUBSTANCES[1]

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The ecological significance of chemoreception to aquatic gastropods and other aquatic invertebrates is well documented (for reviews, see Kohn, 1961; Hodgson, 1955). However, at the molecular level little is known about the substances which influence the behavior of these animals. Blake (1961) attempted to characterize the oyster metabolites which served as attractants to the predatory oyster drill, *Urosalpinx cinerea*. This work was limited primarily to studies on the physical properties of the attractants and to a series of somewhat inconclusive tests with known compounds. Frings and Frings (1965) studied the physical properties of stimulants which diffused from the food of *Aplysia juliana*, but these studies did not include tests with known compounds. Brown and Noble (1960) and Brown (1961) reported that the gastropod, *Bullia laevissima*, would emerge from its buried position when food was near or when certain quaternary amines were present in sufficient concentrations. No attempt was made to correlate the concentrations of compounds necessary for stimulation with the concentrations available when food was placed in the water. Bailey and Laverack (1963), using electrophysiological techniques, reported that receptors in the osphradium of *Buccinum undatum* were sensitive to *Mytilus* extracts as well as to L-glutamic acid and trimethylamine oxide. This work did not include determinations of the relative concentrations of these compounds in the extracts and no reference was made to any response shown by the intact organism.

Nassarius obsoletus was shown by Carr (1967) to be an extremely suitable animal for studies of chemoreception. This marine gastropod displays a stereotyped response which is convenient for measuring the effectiveness of substances extracted from tissues. Carr showed that the principal response-inducing compounds in shrimp extracts were heat-stable, more soluble in polar than in non-polar solvents, non-volatile, of low molecular weight, stable to oxidation, stable to NH_3, and somewhat acid-labile. Further, it was shown that the principle response-inducers were amphoteric and/or weakly basic compounds which were augmented by some acidic and/or neutral compounds(s).

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[1] This paper is based on a portion of a dissertation submitted to the Graduate School of Arts and Sciences of Duke University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Zoology. These studies were conducted at the Duke University Marine Laboratory, Beaufort, N. C., and were supported by a N.I.H. Physiology Training Grant and by a Bureau of Commercial Fisheries Graduate Education Grant. The author is grateful to Dr. K. M. Wilbur, Dept. of Zoology, Duke University, for providing frequent counsel during the course of this research. The fractional ion exchange separation and the quantitative amino acid analyses reported in this paper were conducted in the laboratory of Dr. R. L. Hill, Dept. of Biochemistry, Duke University.

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The foregoing results have been used as the basis for further experiments on chemoreception in *N. obsoletus.* Ion exchange and paper chromatographic techniques were used to identify and subsequently to quantify compounds in shrimp extracts which possessed the established physical and chromatographic properties. Concurrently, an extensive series of tests was performed to determine the stimulatory capacities of compounds and combinations of compounds which were identified in the extracts. In summary, an attempt has been made to account for the response shown by *N. obsoletus* to shrimp extracts, both in terms of the compounds present and their relative concentrations.

Methods

Maintenance of animals, bioassay procedures, and preparation of shrimp extracts

Specimens of *Nassarius obsoletus* were collected, maintained in the laboratory, and tested as previously described (Carr, 1967).

Chloroform:methanol (2:1) extracts of shrimp (*Penaeus duorarum*) were prepared according to the procedure of Folch, Lees and Sloan Stanley (1957). This procedure yields a two-phase system. In each case the upper aqueous phase was removed, evaporated to dryness in an oven at 50° C., suspended in water (1 ml./1 g. initial shrimp weight), and filtered through a Millipore filter. Upper phase material was used extensively and will be designated shrimp extract.

Elution of stimulatory components from paper chromatograms

Sheets of Whatman No. 1 filter paper were prewashed and shrimp extract was applied as a streak to each sheet. A 12-cm. streak was used for 200 μl. of extract; a 15-cm. streak was used for 300 μl. of extract. After development by the descending method, chromatograms were divided into strips (from defined R_f intervals) at right angles to the direction of solvent flow. Each strip was cut to a point and eluted with glass-distilled water for a 12-hour period. Eluates were evaporated to dryness; the residues were suspended in glass-distilled water and filtered prior to testing.

The following solvent systems were employed: glass-distilled water, butanol: pyridine: water (1:1:1) (Smith, 1960, p. 84), propanol:ammonia:water (6:3:1) (Hanes and Isherwood, 1949), and ethanol: ammonia (95:5) (Smith, 1960, p. 84).

Fractional ion exchange separation

A 0.9 × 145 cm. column of Dowex 50-X2 employed in conjunction with a Beckman Spinco Auto-Analyzer (Model 120) was used for fractionation of a shrimp extract. Six ml. of slightly acidified extract were added to the column and a gradient elution was carried out at a flow rate of 1 ml./minute with pH 3.1 pyridine acetate as the starting buffer and pH 5.0 pyridine acetate as the limit buffer (Nelson *et al.,* 1965). The column jacket temperature was 50° C. Fractions of 6 ml. were collected and 0.5-ml. aliquots were treated with ninhydrin reagent (Moore and Stein, 1954). The optical density at 570 mμ was determined for each aliquot and plotted graphically as a function of the fraction number. Fractions contributing to peaks were pooled, concentrated by rotary evaporation *in vacuo,* and each residue was dissolved in 6 ml. of glass-distilled water. Aliquots of each pool were evaporated to dryness in an oven at 50° C. to remove remaining traces of solvent. Residues were redissolved and tested.

Analytical techniques used to identify and quantify compounds in shrimp extracts

Paper chromatography and related techniques. The solvent systems which were used are referred to by the following numbers:

1. Butanol:acetic acid:water (120:30:50) (Smith, 1960, p. 84)
2. Ethanol:ammonia (95:5)
3. Butanol:pyridine:water (1:1:1)
4. Propanol:ammonia:water (6:3:1).

Organic acids were detected with bromcresol green (Smith, 1960, p. 279) or bromphenol blue (Block, Durrum and Zweig, 1958, p. 217). Lactic acid was identified by its R_f values in 4 solvent systems (Nos. 1, 2, 3, and 4).

Betaine and trimethylamine oxide were identified by their reaction with Dragendorff reagent (Bregoff, Roberts and Delwiche, 1953) and by their R_f values in 4 solvent systems (Nos. 1, 2, 3, and 4). Carnitine was tentatively identified by its reaction with Dragendorff reagent and R_f values in two solvent systems (Nos. 1 and 2). Homarine was identified by its UV absorption spectrum, reaction with alkaline alpha-naphthol (Leonard and Macdonald, 1963), and R_f values in two solvent systems (Nos. 1 and 2). Inosine was identified by its UV absorption spectrum and R_f values in 3 solvent systems (Nos. 1, 2, and 3). Urea was identified by its reaction with Ehrlich reagent (Smith, 1960, pp. 193–4) and R_f values in 3 solvent systems (Nos. 2, 3, and 4). Amino acids were identified in the amino acid analyses described below and by two-dimensional chromatography (solvent system No. 1, first dimension: solvent system No. 2, second dimension) using ninhydrin (Smith, 1960, p. 95) as the detection reagent.

Quantitative techniques. Amino acids were analyzed on a Beckman Spinco Auto-Analyzer according to the procedure of Moore, Spackman and Stein (1958) and Spackman, Stein and Moore (1958). Separate analyses were carried out on 100-μl. aliquots of untreated and acid-hydrolyzed (6 N HCl, 20 hours at 110° C.) shrimp extract.

Lactic acid was determined according to the procedure of Barker and Summerson (1941) as modified by Umbreit, Burris and Stauffer (1957, pp. 275–6). A standard curve was prepared using lithium lactate (Amend Drug and Chemical Co.).

Betaine was determined semi-quantitatively by comparing the minimum amount of extracted betaine and betaine-HCl (Calbiochem, A Grade) which could be detected after treatment of chromatograms with Dragendorff reagent. Betaine was also estimated by the visual comparison method of Berry and Cain (1949) as described by Block *et al.* (1958, pp. 86–7).

Preparation of solutions for bioassays of known compounds

For testing a single compound, a concentrated stock solution was prepared in glass-distilled water or in sea water filtered through a Millipore filter; when necessary the pH was adjusted to 6–8 (determined with pHydrion paper). For testing combinations of compounds, concentrated stock solutions were prepared in sea water filtered through a Millipore filter; and the pH was adjusted as above. A solution of the desired concentration was prepared by pipetting a small volume of stock solution into the appropriate volume of sea water. Stock solutions were prepared immediately prior to testing and kept in ice.

N-Acetylglucosamine (A Grade), L-amino acids (A Grade), betaine-HCl (A Grade), glycogen, and Ca-lactate (B-Grade) were obtained from Calbiochem. Na-ascorbate was obtained from Nutritional Biochemicals. Citric acid (Reagent ACS) was obtained from Matheson Coleman and Bell. Glucose (USP) was obtained from Mallinckrodt. Oxaloacetic acid (99.9% purity) was obtained from Sigma. 2-Aminoethylphosphonic acid was supplied by Dr. L. D. Quin, Department of Chemistry, Duke University.

Statistical treatment of data

Many of the data which follow were analyzed by Potency Probit Analysis employing the computer program of Daum and Givens (1963). This program employs the maximum likelihood procedure of Finney (1962). A brief description of the program was given previously (Carr, 1967). The statistical analyses were carried out in the Duke University Digital Computing Laboratory and in the University of Florida Computing Center. The computer program was provided by Mr. Kenneth Fischler, Biometrician, U. S. Fish and Wildlife Laboratory, Beaufort, N. C.

Results

Elution of stimulatory components from paper chromatograms

Paper chromatography was used as one method for the separation of the components in shrimp extracts. After development of chromatograms in one of a series of four solvent systems, substances from defined sectors were eluted with water and bioassayed. Water was used as the initial solvent system to establish whether or not the stimulatory substances moved near the solvent front. If these substances moved near the front, then water could be assumed to be a satisfactory elutant for subsequent experiments. The other solvent systems (butanol:pyridine:water, propanol:ammonia:water, and ethanol:ammonia) were used to take advantage of the differential mobility shown by many compounds in these solvents.

In each experiment, the response obtained from the combination of all sector eluates (*i.e.*, total extract eluate) was used as a basis for determining the relative effectiveness of eluates from specified sectors of the chromatograms. Figure 1 is a diagrammatic representation of the cumulative results of the elution experiments. The stippled regions on the depicted chromatograms portray the R_f intervals from which substances were eluted which possessed stimulatory capacities approaching those of the total extract eluates. These R_f intervals were as follows: water, R_f 80–100; butanol:pyridine:water, R_f 0–60; propanol:ammonia:water, R_f 40–100; ethanol:ammonia, R_f 0–40.

With the exception of chromatograms developed in water in which the majority of substances closely followed the solvent front, it was necessary to combine substances eluted from sizable portions of the chromatograms to obtain solutions with stimulatory capacities approaching those of the total extract eluates. Each of these experiments was repeated and similar results were obtained.

Standards of each of the compounds identified in shrimp extracts (see Table II) were chromatographed in butanol:pyridine:water, propanol:ammonia:water, and ethanol:ammonia in order to relate the R_f values of each of these compounds to the R_f intervals from which the most effective eluates were collected during the experiments cited above. The majority of the compounds had R_f values which placed them within the R_f intervals from which the most effective eluates were collected. With butanol:pyridine:water, only isoleucine, leucine, phenylalanine, tryptophan, and tyrosine had R_f values *not included* in the R_f interval 0–60. With ethanol:ammonia, only homarine, isoleucine, leucine, lactic acid, phenlylalanine, trimethylamine oxide, urea, and valine had R_f values not included in the R_f interval 0–40. With propanol:ammonia:water, all of the compounds had R_f values included in the R_f interval 40–100. These findings suggested that the effectiveness of the eluates collected from the R_f intervals cited above was due to the combined effects of a number of compounds.

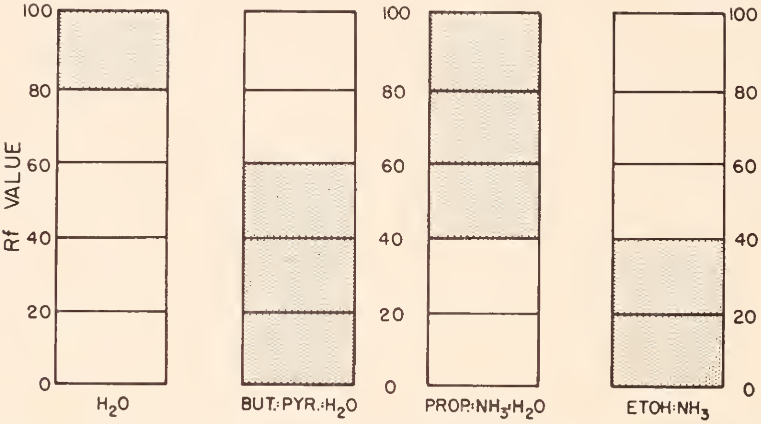

FIGURE 1. Regions of chromatograms from which the principal response-inducing substances in shrimp extracts were eluted. The solvent systems which were employed are given below the diagrammatic paper chromatograms. Stippled regions on the respective chromatograms depict the R_f intervals from which substances were eluted which possessed stimulatory capacities approaching those of the total extract eluates.

Fractional separation of shrimp extract on Dowex 50-X2 column

Previous separations of shrimp extracts on ion exchange columns showed that the principal response-inducers were amphoteric and/or weakly basic compounds which were augmented by some acidic and/or neutral compound(s) (Carr, 1967). A more complete fractionation of shrimp extract was achieved by a fractional separation on a column of Dowex 50-X2. An aliquot of each fraction was treated with ninhydrin reagent and the OD_{570} was determined (Fig. 2). The fractions contributing to the OD peaks were pooled and tested (Table I). Fraction 102 (containing fractions 11–18, stippled peak in Fig. 2) was essentially as effective as the total shrimp extract. This conclusion was supported by a Potency Probit Analysis. Three other fractions (101, 104, and 106) were response-inducing at relatively high concentrations. However, the stimulatory capacities of these latter fractions were very low when compared with the capacity of fraction 102.

Table II summarizes the distribution of the extract components identified in fraction 102 and in other preparations obtained by ion exchange separations which were reported previously (for details of separations of shrimp extracts on columns of Rexyn 102 and Dowex 50W-X8, see Carr, 1967). The water effluent from a column of Rexyn 102 contained all of the compounds identified in shrimp extracts except "carnitine," trimethylamine oxide (TMO), and lysine. Bioassays had revealed that this effluent possessed a stimulatory capacity comparable to that of the total extract. These results implied that carnitine, TMO, lysine, or other such basic compounds, which might be present in the extract, did not contribute appreciably to the stimulatory capacity. The 2 N-NH$_3$ eluate from a column of Dowex 50W-X8 contained all of the compounds identified in the extracts except lactic acid, taurine, and possibly lysine. Bioassays had revealed that this eluate was somewhat less effective than the total extract. The only compounds identified in the water effluent from the column of Dowex 50W-X8 were lactic acid and taurine. Bioassays had revealed that this effluent was only effective at high concentrations when tested alone, but that a combination of the water effluent and the

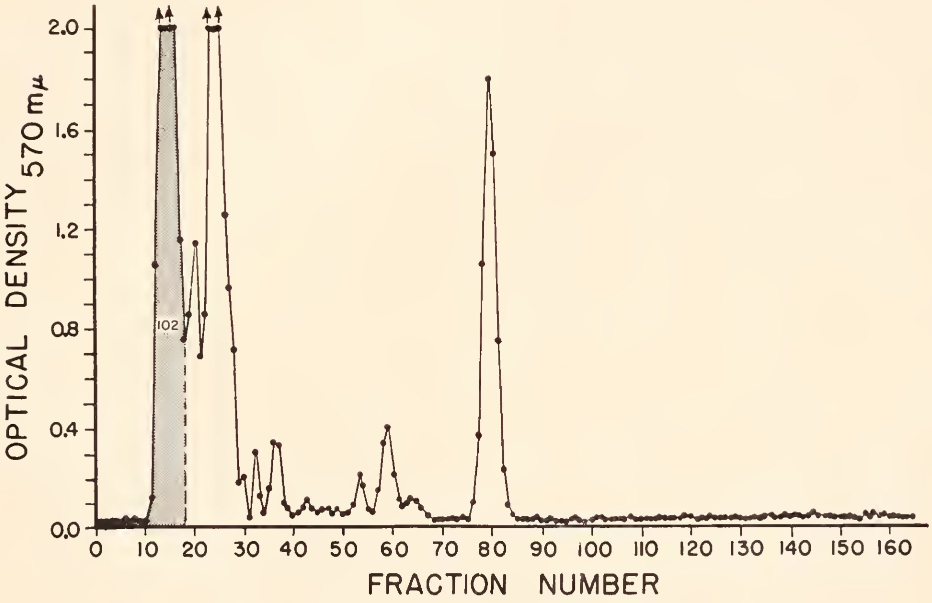

FIGURE 2. Fractionation of a shrimp extract on a column of Dowex 50-X2. Fractions of 6 ml. were collected and aliquots of each were treated with ninhydrin reagent. The optical density at 570 mμ was determined for each aliquot and is plotted as a function of the fraction number. Fractions contributing to peaks were pooled and tested (see Table I).

2 N-NH_3 eluate was as effective as the total extract. These results implied that, although lactate, taurine, and/or some other compound(s) in the water effluent were not the principal source of the extract's stimulatory capacity, they were nevertheless contributors to this capacity. Finally, fraction 102 lacked the following components of the extract: "carnitine," histidine, isoleucine, leucine, lysine, methionine, phenylalanine, TMO, tryptophan, tyrosine, and valine. Bioassays had revealed that this Fraction alone was essentially as effective as the total extract (see Table I). This suggested that the extract's stimulatory capacity was attributable to a "nucleus" of compounds (seemingly represented in Fraction 102) which did not include the members of the list of compounds given immediately above. These results and their implications were investigated as a major portion of the final phase of the problem which is reported later in the Results.

Table I

Responses of N. obsoletus to total shrimp extract and to extract constituents present in fractions collected from column of Dowex 50-X2

Material tested	Column fractions pooled*	Concentration tested (µl./40 ml.)	No. snails tested	% Response
Total shrimp extract		0	20	10
		1	20	15
		2	10	70
		5	20	85
		10	20	70
		20	10	100
Fraction 101	1–10	20	10	30
		60	10	50
102	11–18	2	10	60
		5	30	73
		10	10	60
		20	10	90
103	19–21	60	10	0
104	22–29	20	10	30
		60	10	60
105	30–34	60	10	10
106	35–40	20	10	20
		60	10	40
107	41–50	60	10	20
108	51–56	60	10	0
110	63–75	60	10	10
111	76–85	60	10	10
112	86–115	60	10	10
113	116–165	60	10	20

* The fractions from the Dowex 50-X2 column which contributed to OD_{570} peaks (see Fig. 2) were pooled together. These pools of fractions are referred to as "Fractions" in left hand column of Table.

Quantitative analyses of compounds present in shrimp extracts

Analyses were made to determine the concentrations of the individual amino acids, lactate, and betaine in shrimp extracts. The analyses permitted the testing of artificial solutions which contained various (or all) of these compounds in the same relative concentrations as they occurred in the extracts.

Amino acid analyses. The results of analyses of untreated and acid-hydrolyzed extract are given in Table III. Nineteen amino acids were identified in the untreated extract and 18 were measured quantitatively. Of the latter amino acids, glycine alone accounted for approximately 55% of the total μmoles. Together, glycine, proline (18%), taurine (11%), and alanine (10%) accounted for approximately 94% of the total amino acids in the untreated extract.

TABLE II

Distribution of identified shrimp extract components in stimulatory ion exchange preparations

Compounds identified in shrimp extracts	H_2O effluent from Rexyn 102 column	H_2O effluent from Dowex 50 column	2 N-NH_3 eluate from Dowex 50 column	Fraction 102 from Dowex 50 column
Alanine	+		+	+
Asparagine	+		+	+
Aspartic acid	+		+	+
Betaine	+		+	+
"Carnitine"*			+	
Glutamic acid	+		+	+
Glutamine	+		+	+
Glycine	+		+	+
Histidine	+		+	
Homarine	+		+	+
Inosine	+		+	+
Isoleucine	+		+	
Leucine	+		+	
Lactic acid	+	+		+
Lysine			?	
Methionine	+		+	
Phenylalanine	+		+	
Proline	+		+	+
Serine	+		+	+
Taurine	+	+		+
Threonine	+		+	+
Trimethylamine oxide			+	
Tryptophan	+		+	
Tyrosine	+		+	
Urea	+		+	+
Valine	+		+	
UV-A**	+		+	+

* This compound was tentatively identified as carnitine.
** Unidentified UV-absorbing compound.

Lactic acid analyses. Lactic acid was the only organic acid identified in shrimp extracts. The results of duplicate analyses of lactic acid in two shrimp extracts were as follows:

	μg. lactate/μl. extract
Extract 1	3.95
Extract 2	3.10

The average of these two values (*i.e.* 3.53 μg./μl.) was used in the preparation of stock solutions referred to later in the Results.

Betaine analyses. Semi-quantitative determinations of betaine in shrimp extracts revealed that there were approximately 10 μg. (expressed as betaine-HCl) per μl. of the extracts. This value of 10 μg./μl. was used in the preparation of stock solutions referred to later in the Results.

TABLE III

Amino acids present in shrimp extract

Amino acid	Untreated shrimp extract (μmoles/100 μl.)	Acid-hydrolyzed shrimp extract (μmoles/100 μl.)
Alanine	0.866	0.915
Arginine	—	0.667
Asparagine	0.058*	—
Aspartic acid	0.014	0.072
Glutamic acid	0.049	0.454
Glutamine	0.405*	—
Glycine	4.72	5.34
Histidine	0.064	0.118
Isoleucine	0.064	0.087
Leucine	0.084	0.112
Lysine	0.070	0.033
Methionine	0.083	0.094
Phenylalanine	0.068	0.073
Proline	1.58	1.89
Serine	0.111**	0.111
Taurine	0.938	1.04
Threonine	0.113**	0.113
Tryptophan	Trace	—
Tyrosine	0.052	?
Valine	0.110	0.182

* Calculated from difference between pre- and posthydrolysis values of glutamic acid or aspartic acid.

** Post-hydrolysis value. In analysis of untreated extract, serine, threonine, asparagine, and glutamine came off the column almost simultaneously and their individual concentrations could not be calculated. After hydrolysis, serine and threonine came off as separately definable peaks. Assignment of post-hydrolysis values to amino acids in the untreated extract is subject to a small error but nevertheless provided "approximately correct" values which were used to prepare solutions described later in the Results.

Bioassays of individual compounds

Compounds were selected for testing on the basis of their inclusion in one or more of the following categories: (1) Positive or tentative identification in shrimp extracts. (2) Ubiquitous occurrence at relatively high concentrations in marine animals. (3) Reported to induce feeding responses from other animals. (4) Structural relationship to compound(s) observed to induce responses in *N. obsoletus.*

The most obvious group of compounds identified in shrimp extracts were the amino acids. These identified amino acids were tested individually at concentrations of 10^{-3}, 10^{-4}, and 10^{-5} *M* (unless otherwise specified). Only glycine induced the PSR. The results of tests with glycine are given in Table IV. The concentrations of glycine shown in the Table are all more than 100 times the glycine concentration present in dilutions of shrimp extracts which were stimulatory. Glycine alone was ineffective at the concentrations present in solutions of diluted extract which were capable of marked stimulation.

TABLE IV

Responses of N. obsoletus to known compounds

Compounds identified in shrimp extracts				Compounds not identified in shrimp extracts			
Compound tested	Concentration tested (*M*)	No. snails tested	% response	Compound tested	Concentration tested (*M*)	No. snails tested	% response
Glycine	5.9×10^{-4}	20	25	N-Acetyl-D-glucosamine	1×10^{-4}	10	20
	1.2×10^{-3}	20	45		5×10^{-4}	20	55
	2.4×10^{-3}	20	60		1×10^{-3}	10	50
Lactate	1.0×10^{-4}	20	15	2-Aminoethylphosphonic acid	5×10^{-5}	10	0
	2.5×10^{-4}	20	45		1×10^{-4}	10	40
	5.0×10^{-4}	20	65		5×10^{-4}	10	50
Betaine	1.6×10^{-4}	10	10	Pyruvate	2.5×10^{-4}	20	35
	8.0×10^{-4}	20	25		5.0×10^{-4}	20	50
	1.6×10^{-3}	20	25		1.0×10^{-3}	20	65
	3.2×10^{-3}	10	10				
				Glycogen	0.03 mg./ml.	20	15
					0.06 mg./ml.	20	55
					0.24 mg./ml.	10	80
				D-Glucose	1×10^{-4}	10	0
					1×10^{-3}	10	0
					5×10^{-3}	20	30
					1×10^{-2}	20	30
				Ascorbate (Na)	1×10^{-5}	10	10
					1×10^{-4}	20	20
					1×10^{-3}	10	30
				Citric acid	1×10^{-5}	10	0
					1×10^{-4}	20	30
					5×10^{-4}	20	20
				Oxaloacetic acid	1×10^{-5}	10	0
					1×10^{-4}	20	25
					1×10^{-3}	20	30

Lactate [L (+) Ca-lactate] stimulated the PSR (Table IV) but only at high concentrations relative to extract. The concentrations of lactate shown in the Table are all more than 20 times the lactate concentration present in stimulatory dilutions of shrimp extracts. As with glycine, lactate was ineffective at the concentrations present in solutions of diluted extract which were capable of marked stimulation. Preliminary tests with D(−) lactate revealed that it also was response-inducing but no extensive tests were carried out to compare the stimulatory capacities of the two optical isomers.

Pyruvate (Na) also induced the PSR (see Table IV); it was not identified in shrimp extracts but is mentioned here because of its structural relationship to lactate. A comparison of the results with pyruvate and lactate reveals that the two compounds possessed quite similar stimulatory capacities.

TABLE V

Compounds which were non-stimulatory to N. obsoletus

Amino acids, amines, and related compounds	Range of concentrations tested (M)	Organic acids	Range of concentrations tested (M)
Acetyl choline-Cl	5×10^{-3}–5×10^{-6}	Acetate-Na	5×10^{-4}–10^{-4}
L-Alanine*	10^{-3}–10^{-5}	Fumaric acid	10^{-3}–10^{-5}
γ-Aminobutyric acid	10^{-3}–10^{-5}		
Ammonium-Cl	10^{-3}–10^{-5}	DL-beta-hydroxybutyrate-Na	10^{-3}–10^{-5}
L-Arginine-HCl	10^{-3}–10^{-5}	Malic acid	10^{-3}–10^{-5}
L-Asparagine*	10^{-3}–10^{-5}	Malonic acid	10^{-3}–10^{-5}
L-Aspartic acid*	10^{-3}–10^{-5}	Succinic acid	10^{-3}–10^{-5}
DL-Carnitine-HCl*	10^{-3}–10^{-5}		
Choline-Cl	10^{-3}–10^{-5}	*Carbohydrates*	
L-Citrulline	10^{-3}–10^{-5}		
Deoxycarnitine-HCl	10^{-3}–10^{-5}	D-Glucosamine[00]	5×10^{-4}–10^{-4}
L-Glutamic acid*	10^{-3}–10^{-5}	D-Mannose	10^{-2}–10^{-4}
L-Glutamine*	10^{-3}–10^{-4}	Trehalose	10^{-2}–10^{-5}
Glutathione**	10^{-3}–10^{-5}		
Glycylglycine	10^{-3}–10^{-5}	*Miscellaneous*	
L-Histidine-HCl*	10^{-3}–10^{-5}		
Homarine-SO$_4$*	10^{-4}–10^{-6}		
L-Isoleucine*	10^{-3}–10^{-5}	Adenosine-5-monophosphate[000]	10^{-4}–10^{-6}
L-Leucine*	10^{-3}–10^{-5}		
L-Lysine-HCl*	10^{-3}–10^{-5}	Adenosine-5-triphosphate[000]	5×10^{-4}–5×10^{-5}
L-Methionine*	10^{-3}–10^{-5}	Inosine*	10^{-4}–10^{-6}
Nicotinic acid[00]	10^{-3}–10^{-5}		
L-Phenylalanine*	10^{-3}–10^{-5}	Inosine-5-monophosphate	10^{-4}–10^{-6}
O-Phosphoethanolamine	10^{-3}–10^{-5}	Riboflavin[00]	10^{-4}–10^{-7}
L-Proline*[0]	10^{-3}–10^{-5}		
Sarcosine-HCl	10^{-3}–10^{-5}	Uridine-5-monophosphate	10^{-4}–10^{-6}
L-Serine*	10^{-3}–10^{-5}		
Taurine*	10^{-3}–10^{-5}		
L-Threonine*	10^{-3}–10^{-5}		
Trimethylamine-HCl	10^{-3}–10^{-5}		
Trimethylamine oxide*	10^{-3}–10^{-5}		
L-Tryptophan*	10^{-3}–10^{-5}		
Urea*[00]	10^{-3}–10^{-5}		
L-Valine*	10^{-3}–10^{-5}		

* Compounds identified, or tentatively identified, in shrimp extracts.

** Glutathione (10^{-3}–10^{-6} M) induces a feeding response in *Hydra littoralis* (Loomis, 1955) and *Physalia physalis* (Lenhoff and Schneiderman, 1959).

[0] Proline (10^{-3}–10^{-6} M) induces a feeding reaction in *Cordylophora lacustris* (Fulton, 1963).

[00] Glucosamine (10^{-1}–10^{-5} M), nicotinic acid (10^{-2}–10^{-11} M), riboflavin (10^{-5}–10^{-7} M), and urea (10^{-1}–10^{-3} M) induce ingestion of agar cubes in *Hydra pseudoligactis* (Forrest, 1962).

[000] Adenosine monophosphate (10^{-2}–10^{-5} M) and adenosine triphosphate (10^{-4}–10^{-5} M) induce gorging in the mosquito, *Culex pipiens* (Hosoi, 1959).

Betaine (HCl) was mildly stimulatory but responses of only 25% were obtained with concentrations greater than 100 times the betaine concentration in dilutions of shrimp extracts which were stimulatory. No correlation was apparent between the concentration of betaine tested and the percentage of snails responding.

The relationships between the effective concentrations of shrimp extract and the effective concentrations of glycine, lactate, and betaine are presented graphically at the end of the Results (see Fig. 4).

None of the other compounds identified in shrimp extracts was effective at the concentrations tested. These compounds, and others which were ineffective, are given in Table V.

2-Aminoethylphosphonic acid, N-acetyl-D-glucosamine, and glycogen were not identified in shrimp extracts but were found to possess marked stimulatory capacities (Table IV). D-Glucose, ascorbate (Na), citric acid, and oxaloacetic acid were also not identified in shrimp extracts but were found to be mildly stimulatory (Table IV).

Bioassays of combinations of compounds

Glycine, betaine and lactate were the only compounds identified in shrimp extracts which were stimulatory when tested individually. However, the concentrations of glycine, betaine and lactate in the extracts were much too low to account for the stimulatory capacities of the extracts themselves. The cumulative results of the elutions from paper chromatograms, the separations on ion exchange columns, and the assays of individual compounds revealed that stimulation by shrimp extracts was not due to a single compound and yet not dependent on the presence of every compound in the extracts. Furthermore, stimulation was attributable to amphoteric and/or weakly basic compounds which were augmented by some acidic and/or neutral compound(s).

The constituents of Fraction 102, obtained from the fractional separation of shrimp extract on a column of Dowex 50-X2, possessed a stimulatory capacity comparable to that of the total extract (see Table I) and included a group of identified compounds satisfying the criteria cited above (see Table II). Mixtures of compounds were tested in an attempt to account for the effectiveness of the extracts. Decisions concerning the choice of pertinent compounds to test stemmed from the cumulative results of the many prior tests and yet had as their central focus the results of the fractional separation cited above.

Table VI gives the composition of stock solutions which were used for a number of the bioassays of combinations of compounds. The concentrations given in the Table are the same as the concentrations determined in shrimp extracts (*i.e.,* one μl. of stock solution contains an amount of each component which is comparable to the amount in one μl. of extract).

The amino acids in Solution A, and the betaine (Table VI), were (1) all present in fraction 102, (2) all present in the water effluent from a column of Rexyn 102, (3) all, except taurine, present in the 2 N-NH_3 eluate from a column of Dowex 50W-X8 (taurine was present in the water effluent), and (4) all present in the R_f intervals, 0–40 (solvent, ethanol:NH_3), 40–100 (solvent, propanol:NH_3:water), and 0–60 (solvent, butanol:pyridine:water). Lactate was (1) present in fraction 102, (2) present in the water effluent from a column of Rexyn 102, (3) present in the water effluent from a column of Dowex 50W-X8, and (4) present in the aforementioned R_f intervals in the solvents propanol:NH_3:water and butanol: pyridine:water, but had an R_f of 52 in ethanol:NH_3.

The following studies were carried out over a 3-month period during the summer (1965) because of indications of a seasonal variation in responsiveness (unpublished personal observations). It was apparent that snails collected during the warm months were somewhat more responsive than snails collected during the cold months.

Tests of Solution A, betaine, and lactate, and glycine, betaine, and lactate. Figure 3 contains the results of tests of Solution A (glycine + 9 other amino acids), betaine, and lactate, and of glycine, betaine, and lactate. The combination of Solution A, betaine, and lactate was very effective and possessed a stimulatory capacity considerably greater than was attributable to the concentrations of glycine, betaine, and lactate which were present in it. With the exception of glycine, the amino acids in Solution A were *not* effective when tested individually even at high concentrations (10^{-3} *M*). However, when combined with glycine, betaine, and lactate, these amino acids made a marked contribution to the stimulatory capacity. The Solution A-betaine-lactate combination was approximately 10–13 times as effective as either lactate or glycine-betaine-lactate and approximately 37 times as effective as glycine. The glycine-betaine-lactate combination was no more effective than lactate alone. Lactate and glycine-betaine-lactate were both significantly more effective than glycine.

TABLE VI

Composition of stock solutions used for bioassays of combinations of compounds

Stock solution	Compounds included	Concentration (mg./ml.)
Solution A (amino acids)	L-Alanine	0.77
	L-Asparagine	0.09
	L-Aspartic acid	0.02
	L-Glutamic acid	0.07
	L-Glutamine	0.59
	Glycine	3.54
	L-Proline	1.82
	L-Serine	0.12
	L-Taurine	1.18
	L-Threonine	0.14
Betaine solution	Betaine-HCl	10.0
Lactate solution	L(+) Ca-Lactate	5.92

Tests of other combinations of Solution A, betaine, and lactate. In an attempt to gain insight into the relative importance of betaine and lactate in the Solution A-betaine-lactate combination, solutions were tested which lacked betaine and/or lactate. The results are given in Table VII. Solution A was markedly less effective than combinations of Solution A with betaine or lactate. At concentrations of 250, 500, and 1000 μl./40 ml., Solution A induced responses of 40%, 50%, and 40%, respectively, whereas at concentrations of only 25, 50, and 100 μl./40 ml., Solution A-lactate induced responses of 42%, 60%, and 55%, respectively, and Solution A-betaine induced responses of 20%, 43%, and 47%, respectively. The Solution A-lactate combination was seemingly more effective than the Solution A-betaine combination; however, a Potency Probit Analysis showed that neither of the latter combinations was significantly less effective than the Solution A-betaine-lactate combination. Nevertheless, the difference in the calculated potencies of Solution A-betaine-lactate and Solution A-betaine (no lactate) was nearly significant; the upper and lower 95% confidence limits were 1.19 and 0.22, respectively (see Table VII). During the bioassays this difference seemed very real and it was felt that the combination which *excluded* lactate was *less* effective than either Solution A-betaine-lactate or Solution A-lactate. The role of betaine was

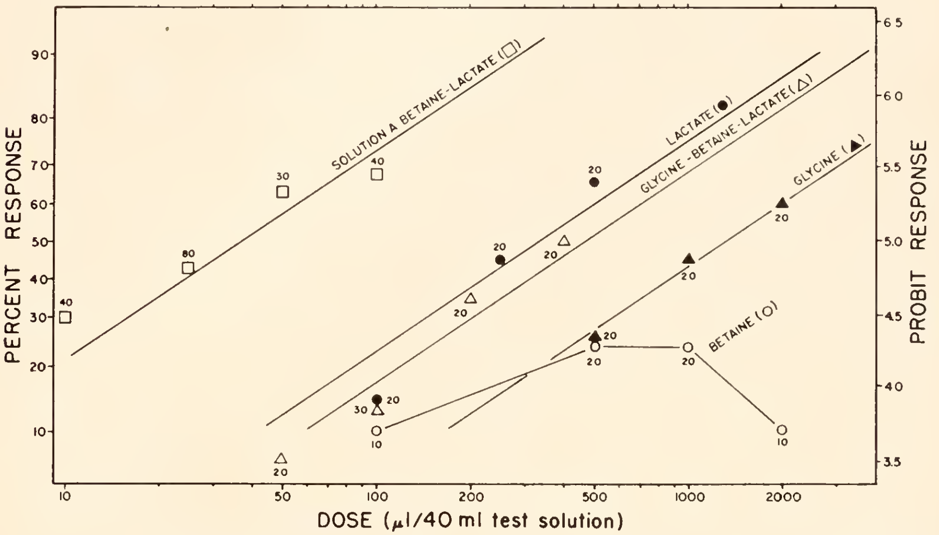

FIGURE 3. Results of bioassays of certain compounds identified in shrimp extracts. Per cent response (left ordinate) and probit response (right ordinate) are plotted as a function of dose (abscissa-log$_{10}$ scale). The experimental dosage-response values are indicated by symbols; numbers next to symbols indicate numbers of snails tested. Regression lines were drawn from computed effective dose values obtained by Potency Probit Analysis. No regression line was computed for results obtained with betaine. The potencies (and 95% confid. lims.) given below were computed with respect to the potency of Solution A-betaine-lactate:

	Upper lim.	0.141
Glycine-betaine-lactate	Potency	0.079
	Lower lim.	0.036
	Upper lim.	0.206
Lactate	Potency	0.109
	Lower lim.	0.056
	Upper lim.	0.052
Glycine	Potency	0.028
	Lower lim.	0.014
Betaine	Not computed	

uncertain; Solution A-betaine was considerably more effective than Solution A alone but Solution A-lactate (no betaine) was essentially as effective as Solution A-betaine-lactate.

Tests of Solution A, betaine, and lactate plus additional compounds identified in shrimp extracts. Histidine, isoleucine, leucine, lysine, methionine, phenylalanine, trimethylamine oxide, tryptophan, tyrosine, urea, and valine were other compounds identified in shrimp extracts. In order to determine whether these compounds contributed to the response-inducing capacity of the extracts, these compounds were incorporated into a single stock solution and tested in combination with Solution A-betaine-lactate. The relative concentration of each compound was equal to the concentration determined in shrimp extracts. Incorporation of these additional compounds yielded a solution which was no more effective than a solution containing only Solution A, betaine, and lactate. This conclusion was supported by a Potency Probit Analysis. The implication that histidine, isoleucine, leucine, lysine, methionine, phenylalanine, trimethylamine oxide, tryptophan, tyrosine, and valine were not among the principal contributors to the stimulatory capacity of the extracts was suggested initially by the fact that these compounds were not identified in fraction 102 (see Table II).

TABLE VII

Responses of N. obsoletus to Solution A, Solution A-betaine, and Solution A-lactate

Solution A* (μl./40 ml.)	Betaine* (μl./40 ml.)	Lactate* (μl./40 ml.)	No. snails tested	Per cent response	Potency** (95% confid. lims.)
125	—	—	10	20	Not computed
250	—	—	20	40	
500	—	—	20	50	
1000	—	—	10	40	
25	25	—	10	20	Up. lim. 1.198
50	50	—	30	43	Potency 0.489
100	100	—	30	47	Low. lim. 0.219
200	200	—	30	73	
10	—	10	40	35	Up. lim. 2.124
25	—	25	60	42	Potency 1.030
50	—	50	40	60	Low. lim. 0.509
100	—	100	40	55	

* Stock solutions prepared as given in Table VI.

** Potencies (and confid. lims.) computed with respect to the potency of Solution A-betaine-lactate. Data from tests of the latter combination are given in Figure 3.

Summary—Relative effectiveness of shrimp extract and certain components in shrimp extract. Figure 4 permits a more adequate visualization of the approach made to the response-inducing capacity of shrimp extract as a result of the systematic incorporation of extract components. The complete response-inducing capacity of the extract was not attained with the combinations of compounds tested; the extract was approximately 6 times as effective as the Solution A-betaine-lactate combination. Nevertheless, the results very strongly implied that the extract's stimulatory capacity stemmed from a group of compounds. Solutions containing only Solution A (10 amino acids), glycine, betaine, *or* lactate (or glycine-betaine-lactate) were considerably less effective than Solution A-betaine-lactate. The effectiveness of the Solution A-betaine-lactate combination was *not* simply a function of the presence of more organic molecules; actually there were considerably fewer molecules present in the stimulatory solutions produced from combining Solution A, betaine, and lactate than in the less effective solutions containing only certain of these constituents.

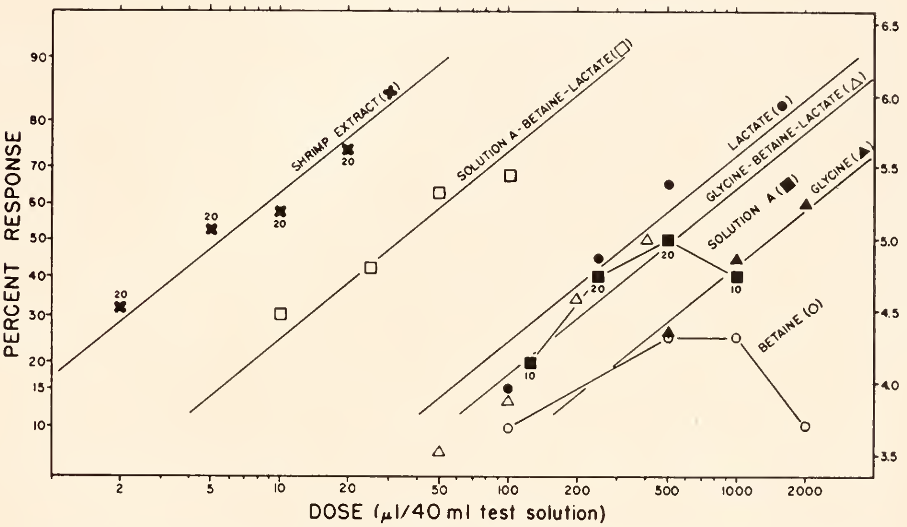

FIGURE 4. Responses given by *N. obsoletus* to a shrimp extract and to certain compounds identified in shrimp extracts. Per cent response (left ordinate) and probit response (right ordinate) are plotted as a function of dose (abscissa-$\log_{10}$ scale). The experimental dosage-response values are indicated by symbols. Numbers next to symbols for tests of shrimp extract and Solution A indicate numbers of snails tested; numbers of snails tested with other preparations were given in Figure 3. Regression lines were drawn from computed effective dose values obtained by Potency Probit Analysis. No regression lines were computed for results obtained with Solution A or betaine. The potencies (and 95% confid. lims.) given below were computed with respect to the potency of shrimp extract:

	Upper lim.	0.313
Soln. A-betaine-lactate	Potency	0.166
	Lower lim.	0.084
	Upper lim.	0.035
Lactate	Potency	0.016
	Lower lim.	0.006
	Upper lim.	0.023
Glycine-betaine-lactate	Potency	0.011
	Lower lim.	0.004
	Upper lim.	0.009
Glycine	Potency	0.004
	Lower lim.	0.002
Soln. A	Not computed	
Betaine	Not computed	

DISCUSSION

By using the proboscis search reaction of *Nassarius obsoletus* as the criterion of response, it has been possible to describe three aspects of stimulation by tissue extracts: (1) individual stimulatory compounds were identified; (2) the effectiveness of these compounds was measured as a function of their concentrations in the extracts; and (3) mixtures of compounds were shown to be more effective than the individual compounds in the mixtures.

The approach taken by previous workers on related problems with aquatic invertebrates was confined primarily to the identification of specific stimulatory compounds (*cf.* Loomis (1955) with *Hydra*; Lenhoff and Schneiderman (1959) with *Physalia*; Brown (1961) with *Bullia*; and Fulton (1963) with *Cordylophora*). No attempts were made to show that the concentration of the identified response-inducer(s) in tissue preparations was sufficient to account for the responses observed to the preparations themselves. Moreover, in the present study a chemically mediated response has been shown to involve a group of compounds whose members complement one another in a manner unsuspected on the basis of tests of these individual members.

All of the compounds included in the studies of stimulation by combinations of compounds were identified constituents of shrimp extracts and are representative of the compounds found in the tissues of other marine animals. Free amino acids and betaine occur in high concentrations in marine invertebrates (Awapara, 1962; Laverack, 1963) and marine vertebrates (Shewan, 1962). Lactic acid is a ubiquitous muscle constituent which accumulates as a result of muscular activity (White *et al.,* 1959, pp. 783–787) and post-mortem glycolysis (Tomlinson *et al.,* 1963). Furthermore, certain basic similarities in the chemical composition of many marine animals may account in part for the fact that *N. obsoletus* is not a "selective scavenger."

The reported experiments were confined to compounds found in fresh tissues. Products of bacterial decomposition may be equally stimulatory to this snail. Preliminary experiments revealed that the stimulatory capacity of a casein hydrolysate (prepared in sea water) increased considerably after standing at room temperature for 24 hours; bacterial activity was apparent because the solution became cloudy and developed a strong stench. However, it was considered that a greater contribution could be made by directing efforts toward the identification of endogenous tissue components which are themselves extremely effective response-inducers.

Of the compounds identified in shrimp extracts, only glycine (*ca.* 10^{-3} *M*) and lactate (*ca.* 5×10^{-4} *M*) possessed marked stimulatory capacities when tested individually. A third extract component, betaine (*ca.* 10^{-3} *M*), was mildly stimulatory. 2-Aminoethylphosphonic acid (*ca.* 5×10^{-4} *M*), N-acetylglucosamine (*ca.* 5×10^{-4} *M*), glycogen (*ca.* 0.06 mg./ml.), and pyruvate (*ca.* 5×10^{-4} *M*) possessed marked stimulatory capacities but were not identified in shrimp extracts. Henschel (1932) reported that glycine (*ca.* 10^{-3} *M*), lactate (*ca.* 10^{-2} *M*), and glycogen (*ca.* 0.06 mg./ml.) induced extensions of the proboscis by *Nassarius reticulatus*. It is noteworthy that these three compounds induce the same type of response in two related gastropods, one a North American species (*N. obsoletus*) and the other a European species (*N. reticulatus*). Trimethylamine (*ca.* 10^{-5} *M*), reported by Brown (1961) to be stimulatory to the gastropod, *Bullia laevissima,* did not induce the PSR in *N. obsoletus* when tested at comparable concentrations.

The role of the osphradium in chemoreception by gastropods was first demonstrated by Copeland (1918) and later confirmed by Brown and Noble (1960). Bailey and Laverack (1963) found that stimulation of the osphradium of *Buccinum undatum* with *Mytilus* extracts, L-glutamic acid (*ca.* 10^{-3} *M*), and trimethylamine oxide (*ca.* 10^{-2} *M*) resulted in volleys of action potentials in the central nervous system. L-glutamic acid and trimethylamine oxide were not response-inducing in *N. obsoletus* at concentrations of 10^{-3} to 10^{-5} *M*. Electrophysiological techniques have been employed to study the sensitivity of chemoreceptors in marine arthropods to various compounds including glycine and betaine which induce the PSR in *N. obsoletus*. Barber (1961) reported that *Limulus polyphemus* possessed gnathobase receptors sensitive to glycine (*ca.* 10^{-3} *M*). Case and Gwilliam (1961) and Case (1964) found that dactyl receptors in several species of decapods are sensitive to a variety of amino acids (including glycine, *ca.* 5×10^{-2} *M*). Laverack (1963) showed that three species of decapods possess dactyl receptors sensitive to betaine (*ca.* 10^{-2} *M*) and trimethylamine oxide (*ca.* 10^{-2} *M*), but insensitive to glycine and several other amino acids. Levandowski and Hodgson (1965) found that the spiny lobster, *Panulirus argus*, possesses receptors on the antennules and dactyls which are sensitive to betaine, L-glutamic acid, trimethylamine, and trimethylamine oxide at concentrations of 10^{-3} *M*. These receptors were somewhat less sensitive to glycine (*ca.* 10^{-2} *M*). The possession of receptors sensitive to amino acids and/or other nitrogenous compounds of low molecular weight is characteristic of the marine arthropods and molluscs which have been studied.

Compounds with slight differences in molecular structure possessed markedly different stimulatory capacities in *N. obsoletus*. Of the 20 amino acids (19 L-amino acids and glycine) bioassayed over a concentration range of 10^{-3}–10^{-5} *M*, only glycine induced responses. Molecular modifications in the glycine (H_2NCH_2COOH) moiety, as represented by the other amino acids ($H_2NCHRCOOH$), sarcosine (H_3CNHCH_2COOH), and glycylglycine ($H_2NCH_2CONHCH_2COOH$), resulted in a loss of stimulatory capacity at the concentrations at which glycine was effective (*ca.* 10^{-3} *M*).

Lactate ($CH_3CHOHCOO^-$) and pyruvate (CH_3COCOO^-) each induced a response of approximately 50% at 5×10^{-4} *M*; oxaloacetate ($^-OOCCH_2COCOO^-$) was less effective than either, while acetate (CH_3COO^-), beta-hydroxybutyrate ($CH_3CHOHCH_2COO^-$), malate ($^-OOCCH_2CHOHCOO^-$), and malonate ($^-OOCCH_2COO^-$) were ineffective at comparable concentrations. Again slight changes in molecular configurations resulted in marked changes in stimulatory capacity.

2-Aminoethylphosphonic acid ($H_2NCH_2CH_2PO_3H_2$), reported by Quin (1965) to occur in at least three marine phyla, induced a response of 50% at 5×10^{-4} *M*; phosphoethanolamine ($H_2NCH_2CH_2OPO_3H_2$) was ineffective. The molecular difference in this case involved a C-P (a phosphonic acid) bond and a C-O-P (a phosphoryl ester) bond. N-acetylglucosamine induced a response of approximately 50% at 5×10^{-4} *M*; glucosamine was ineffective. The acetyl radical on the amino group of the former compound was the only molecular difference involved.

Since it was not the intent of this research to explore thoroughly the relative effectiveness of related compounds, the numbers and concentrations of tested compounds were limited. Nevertheless, the findings that closely related compounds showed very different stimulatory capacities were suggestive of considerable receptor specificity. This aspect of chemoreception in *N. obsoletus* will be studied in more detail in the future.

Each solution prepared from an ion exchange or a paper chromatographic separation of a shrimp extract, which possessed a stimulatory capacity comparable to that of the total extract, was found to contain certain amino acids, *plus* betaine and lactate. Of these compounds, glycine, betaine, and lactate were each stimulatory when tested individually. However, the extracts themselves were effective at concentrations much lower than was attributable to the concentrations of glycine, betaine and lactate present (see Fig. 4). Moreover, a combination containing these three compounds in the same relative concentrations as they occurred in the extracts was no more effective than lactate alone. None of the nine amino acids, alanine, asparagine, aspartic acid, glutamic acid, glutamine, proline, serine, taurine, or threonine, was observed to be stimulatory when tested individually even at high concentrations (10^{-3} M). However, a combination containing these nine amino acids and glycine (Solution A), plus betaine and lactate, was considerably more effective than any of the individual compounds, or Solution A, or a combination containing only glycine, betaine, and lactate (see Fig. 4).

The effectiveness of the Solution A-betaine-lactate combination was due to an apparent synergistic effect. The fact that the stimulatory capacity of this combination was approximately 10–13 times as great as that of the glycine-betaine-lactate combination was not due to the presence of more total solute (*i.e.,* more organic molecules). The dose of Solution A-betaine-lactate which was sufficient to induce a response of approximately 50% contained only approximately 0.09 times as many micromoles of solute as the dose of glycine-betaine-lactate which was necessary to induce a comparable response. Hence the contributions of the components in the Solution A-betaine-lactate combination were not simply additive.

The *complete* stimulatory capacity of the shrimp extracts was not attained with the combinations of compounds which were tested. These combinations obviously lacked a component(s) which contributed to the effectiveness of the extracts themselves. Nevertheless, the results of these studies provide insight into factors which were difficult to consider in previous studies of chemoreception by aquatic invertebrates. The observation that certain compounds in an extract are stimulatory when tested individually does not justify the assumption that the stimulatory capacity of the extract resides entirely in these compounds. In this study, three compounds in a shrimp extract were found to be stimulatory when tested individually; however, these compounds were shown to be considerably less effective than the extract itself. Likewise, the observation that other compounds in an extract are individually non-stimulatory does not justify the assumption that such compounds make no contribution to the extract's stimulatory capacity. In this study, compounds in a shrimp extract which were individually non-stimulatory were found to contribute to the response-inducing capacity of a mixture of compounds.

SUMMARY

1. A study was made of the compounds in shrimp extracts which induce the proboscis search reaction in *Nassarius obsoletus*.

2. Compounds identified in shrimp extracts were as follows: alanine, asparagine, aspartic acid, betaine, glutamic acid, glycine, histidine, homarine, inosine, isoleucine, leucine, lactic acid, lysine, methionine, phenlyalanine, proline, serine, taurine, threonine, trimethylamine oxide, tryptophan, tyrosine, urea, and valine. Carnitine was tentatively identified. The amino acids and lactic acid were determined quantitatively; betaine was determined semi-quantitatively.

3. Glycine (*ca.* 10^{-3} *M*) and lactate (*ca.* 5×10^{-4} *M*) were the only compounds identified in the extracts which possessed marked stimulatory capacities when tested individually; betaine (*ca.* 10^{-3} *M*) was mildly stimulatory. However, quantitative analyses of these compounds in shrimp extracts showed that they were present in insufficient concentrations to account for the responses observed with the dilutions of extract which were employed.

4. Elutions of extract components from paper chromatograms revealed that eluates from large portions of chromatograms were more effective than eluates from small portions. This implied that the stimulatory capacity of the total extract stemmed from the combined effect of a number of compounds.

5. The response-inducing capacity of a combination of twelve compounds identified in the extracts (glycine, lactate, betaine, alanine, asparagine, aspartic acid, glutamic acid, glutamine, proline, serine, taurine, and threonine) was greater than was attributable to the response-inducing capacities of the individual compounds. This combination of compounds possessed a stimulatory capacity which approached, though it did not attain, the stimulatory capacities of shrimp extracts. The effectiveness of this combination of compounds was not increased by the combined addition of histidine, isoleucine, leucine, lysine, methionine, phenylalanine, trimethylamine oxide, tryptophan, tyrosine, urea, and valine.

6. N-Acetylglucosamine (*ca.* 5×10^{-4} *M*), 2-aminoethylphosphonic acid (*ca.* 5×10^{-4} *M*), glycogen (*ca.* 0.06 mg./ml.), and pyruvate (*ca.* 5×10^{-4} *M*) also possessed marked response-inducing capacities in *N. obsoletus*. Ascorbate (*ca.* 10^{-4} *M*), citric acid (*ca.* 10^{-4} *M*), glucose (*ca.* 5×10^{-3} *M*), and oxaloacetic acid (*ca.* 10^{-4} *M*) were mildly stimulatory.

7. Compounds structurally related to N-acetylglucosamine, 2-aminoethylphosphonic acid, glycine, lactate, and pyruvate were either less effective or ineffective when tested at comparable concentrations. These findings suggest that considerable receptor specificity may exist.

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CHEMORECEPTION IN CIRRIPEDES

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In reviewing the different functions of cirral activity in barnacles, Crisp and Southward (1961) distinguished captorial feeding, in which larger food particles were caught in the cirral net, from microphagy, in which small particles were filtered from the water currents entering the mantle cavity.

They found a number of differences between the action of the cirri of the more primitive Thoracica, the stalked barnacles or *Lepadidae,* and that of the most advanced sessile forms, the *Balanidae.* In the *Lepadidae,* as Barnes and Reese (1959) had previously demonstrated with *Pollicipes polymerus,* the cirri sometimes roll up independently and sometimes together, whereas the cirri of the *Balanidae* almost invariably roll up synchronously, enclosing the prey in a compact cirral net. The *Lepadidae* usually rely on external water movements to bring food into the cirral net. They display less specialized forms of cirral activity, extending the cirri passively against the flow of water until food particles are caught.

The *Balanidae* display a greater variety of feeding mechanisms including the active capture of larger prey in the course of rhythmic cirral activity and the filtration of fine particles with the cirral net wholly or partially extended. With certain exceptions, members of the *Lepadidae* are less sensitive to mechanical shock and remain closed afterwards for shorter periods than members of the *Balanidae.*

Crisp and Southward offered evidence that some of the *Balanidae* were able to select between nutritious and non-nutritious particles caught on the extended cirral net. Cellulose fibers, air bubbles, and other inert particles were ignored, but when food particles touched the cirral net, the cirri closed on them and transferred them to the mouth. Food particles were not only ingested to a greater extent, but the presence of food in the water in the form of small plankton or finely chopped muscle tissue appeared to stimulate more active and continuous feeding. This effect was later demonstrated by continuously recording the cirral movements of the barnacle *Elminius modestus* (Southward and Crisp, 1965). An ability to respond to chemical substances dissolved in the surrounding water or diffusing from captured food material impaled on the cirri seems the most likely means by which food is selected on capture. It is interesting to note that in the cinematographic record of the capture of a food particle (Crisp and Southward, 1961; Fig. 14), there was a delay of 0.3–0.5 sec. after the particle had struck the cirrus before the closing action began. Less delay would have been expected for a direct response to a mechanical stimulus than for a response brought about by diffusion of material from the food particle to a receptor.

Barnes and Reese (1959) reached quite different conclusions from observations on the behavior of the stalked barnacle *Pollicipes polymerus.* They found that lightly touching the inner surface of the flagellum of the cirrus with a needle caused it to flex independently. Stronger or repeated stimuli led to a graded response in which more and more of the cirri bent over the needle, culminating in a typical captorial enclosure by the whole cirral apparatus. They suggested that repeated mechanical stimuli caused by the struggling movements of the prey, like those evoked by the needle, progressively reinforced each other and resulted in capture of the prey and its transfer to the mouth. Such an experiment cannot readily be performed on most balanomorph species because touch so readily elicits a shock reaction. Barnes (1959) observed both organic and inorganic particles in the stomach contents of several different species of acorn barnacles, from which he concluded that there was little selection. Nevertheless he thought a chemotactic sense determined whether particles, after having been manipulated in the oral region, were later swallowed or rejected. From an examination of the stomach contents of *Pollicipes polymerus* he obtained evidence of selection in favor of animal prey, but he ruled out the likelihood that chemosensation played any part and considered the distinction between living prey and sand particles was entirely mediated by mechanoreceptors.

The experiments described below were carried out to determine whether a purely chemical stimulus can elicit feeding response in cirripedes. Pedunculate forms were used since Barnes formed his views largely as a result of experimenting with one such species.

In this paper the phrase "chemical stimulation" is used to describe movements, apparently concerned with food capture, induced by chemical substances dissolved in the water. The inhibitory or narcotic effect of chemicals causing reduction in cirral activity, though referred to by Cole and Allison (1930, 1932, 1933, 1937) as "chemical stimulation," is not considered relevant to this study.

Methods

Two species of pedunculate barnacles were used for the majority of investigations, *Lepas anatifera* and *Lepas fascicularis*. The former was found attached to pieces of floating wood, the latter to floating fronds of *Fucus vesiculosus*. *Lepas anatifera* was found to be the more robust and reliable species for experimental work, and quantitative studies were confined to it. Qualitative observations were usually replicated on *Lepas fascicularis* with results closely similar to those found with *L. anatifera*.

The animals were maintained in a large tank of about 50 L. capacity in which the water was kept flowing continuously. They were fed each evening on scraps of *Mytilus edulis*.

Tests for chemosensory behavior were made as follows. About 0.3 ml. of test solution was sucked into an Aglar micro syringe fitted with a fine hypodermic needle and a micrometer screw-driven piston which allowed deliveries of solution to an accuracy of ± 0.0005 cc. to be made without difficulty. The tip of the needle was held about 3 cm. from a selected individual, and directed towards part of its open cirral net, usually about half-way along the flagellum of a cirrus. The exact position seemed unimportant, and similar results were obtained if the needle was directed at the mouth parts. A jet of solution of 0.01 cc. was then squirted on to the cirrus by turning the micrometer head through 360°. The solution was considered to have caused a response if the cirrus rolled up, whether independently or as part of a general contraction of the cirral apparatus. Typically, a strong stimulus caused the whole set of larger cirri immediately to close over the mouth and to draw themselves several times over the shorter cirri which, meanwhile, executed rapid reciprocating movements as though forcing food towards the oral cone. When a partial closure involving only a few cirri was observed it was designated a weak response.

Whenever positive responses were observed, a control observation was made by squirting 0.01 cc. of sea water over the same animal. Only rarely did this evoke any response, and then only a partial flexure of an individual cirrus, which was not usually repeated when sea water was squirted again. Solutions giving a true feeding reaction, in contrast, gave consistent and repeatable responses, usually by more than a single cirrus.

Failure to produce a response by a given solution was confirmed by squirting at the same individual in place of the solution, a suspension of one part of milk in three parts of sea water; this invariably caused a response.

Quantitative measurements of the threshold of sensitivity were made by serial dilution of the test chemical by a factor of 1.5 or 2 times. At each concentration, some 20 tests were made and the percentage of positive responses counted. The threshold value was taken as the point at which 50% of individuals failed to give the response.

Except where otherwise stated, solutions were made up in laboratory sea water, and tested for neutrality before use.

Observations were originally made on *Lepas anatifera* washed up at Woods Hole. Later, a population washed up on the west coast of Anglesey was tested using glycine, and gave identical results (Fig. 3).

PRELIMINARY EXPERIMENTS

Initially, a number of naturally occurring and pure substances were applied to the cirri to indicate the kind of material likely to be worthy of quantitative investigation. Milk, bacto-peptone, coelomic fluids of animals, and tissue extracts invariably gave rise to strong feeding responses. Solutions of proteins, such as gelatin and pepsin, caused rather weak reactions which became even less definite after dialysis, but several amino acids, such as glycine, alanine, glutamic acid, arginine, proline, and serine, gave a clear positive response, while the peptide, reduced glutathione (at 1.6×10^{-2} *M*), and solutions of the larger amino acids gave generally weak feeding reactions. Allantoin failed to give a definite response at all.

Sugars such as sucrose, glucose, galactose and lactose, and cultures of *Phaeodactylum* and *Dunaliella* were all without effect; a strong suspension of soluble starch and a solution of glycogen elicited only very weak responses.

Organic substances in general caused no reaction. Sodium acetate and caproate (1.6×10^{-2} *M*), sodium benzoate (5×10^{-2} *M*) and stearate, ethyl alcohol (10%), sea water saturated in aniline, and tetramethylammonium chloride were tested, all with negative results.

Certain substances that altered the pH of sea water through hydrolysis caused an apparent feeding reaction accompanied by a withdrawal movement of the whole animal brought about by bending the stalk. The latter reaction was evident when strong solutions of aspartic acid and cysteine which were rather far from neutrality were used, and also on application of certain amino acids in neutral solution (see below). Similar stalk-bending reactions, as well as feeding reactions, were observed when sea water was made acid or alkaline and squirted over the animals. The range of pH which did not elicit any response was found to lie between pH 7.5 and 9.5, and any test solutions whose pH did not fall well inside these limits were discarded. Sodium bicarbonate at 0.5 *M* was found to give satisfactory

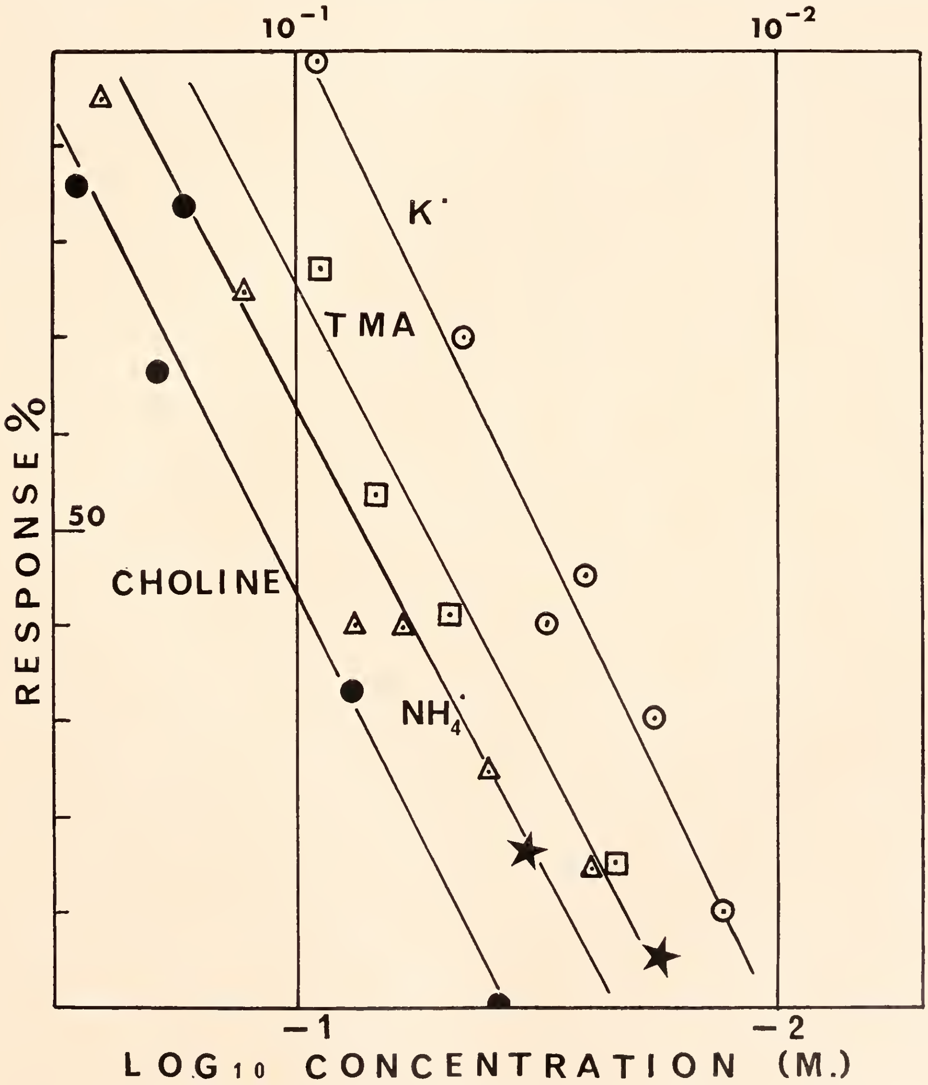

FIGURE 1. Reaction of *Lepas anatifera* to solutions of various cations in neutral solution in sea water, buffered where necessary with carbonate. Potassium: open circles. Trimethylamine: squares. Trimethylamine oxide: stars. Ammonium: triangles. Choline: full circles.

buffering for most of the more acid solutes and did not itself produce any reaction. Distilled water produced a shock reaction, causing the animal to close temporarily, but sea water diluted by 50% had no effect.

Responses to Inorganic Ions

In addition to the influence of the hydrogen ion, the effect of solutions of other inorganic cations was tested by mixing solutions of salts with sea water in varying proportions. Of the commoner salts, only those with monovalent cations evoked any response, and then only at concentrations exceeding 10^{-2} *M*. Since the sodium ion is present in such high concentration in sea water, it was not expected to have any effect, and indeed equimolar (0.5 *M*) solutions of sodium chloride or sodium bicarbonate had none. Hypertonic solutions (1.0 *M*) of sodium chloride did, however, evoke a regular response. Ammonium salts (chloride, sulfate and nitrate) neutralized with sodium bicarbonate, and potassium salts brought about a feeding reaction at much lower concentrations, as shown in Figure 1. The figure shows that, within the 10 and 90 percentile values of the number of animals responding in the sample population, an approximately linear relationship exists between the

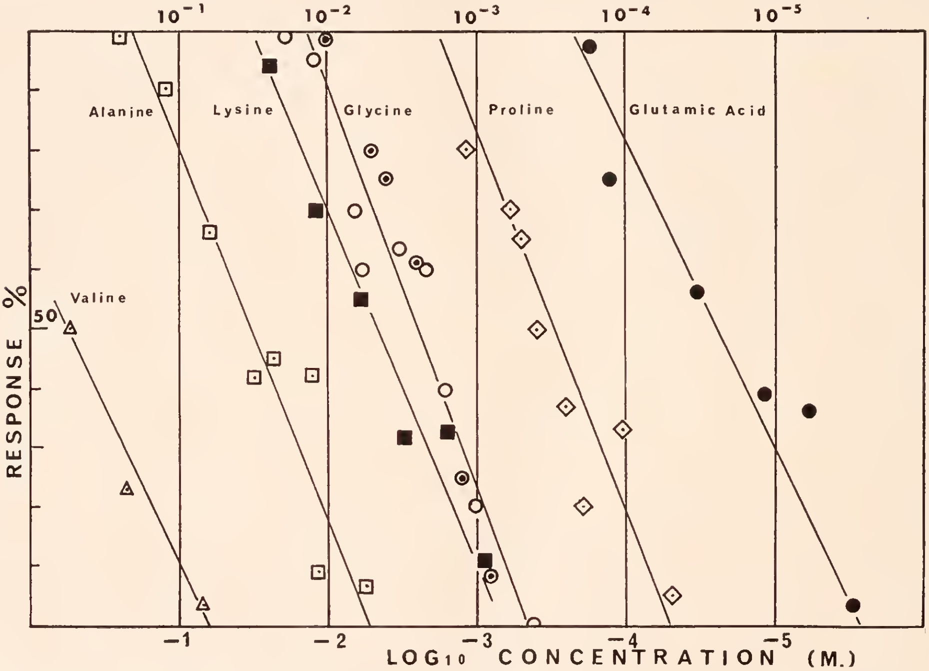

Figure 2. Reaction of *Lepas anatifera* to solutions of amino acids in neutral solution in sea water, buffered where necessary with carbonate. L-valine: triangles. L-alanine: open squares. L-glycine, Woods Hole population of *Lepas:* open circles. L-glycine, North Wales population of *Lepas:* ringed circles. L-proline: diamonds. L-lysine: full squares. L-glutamic acid: full circles.

percentage response, and the logarithm of the concentration applied. The concentration at the intercept with a response by 50% of the population is taken to characterize the population threshold for the substance. This was 6.6 and 3.0 × 10^{-2} *M*, respectively, for ammonium and potassium. Calcium, strontium and magnesium had no influence when used at strengths of 0.5 *M*. However, when magnesium and potassium ions were present in equal amounts, the effect of the potassium ion was slightly reduced, indicating antagonism. Inorganic anions appeared to be quite ineffective.

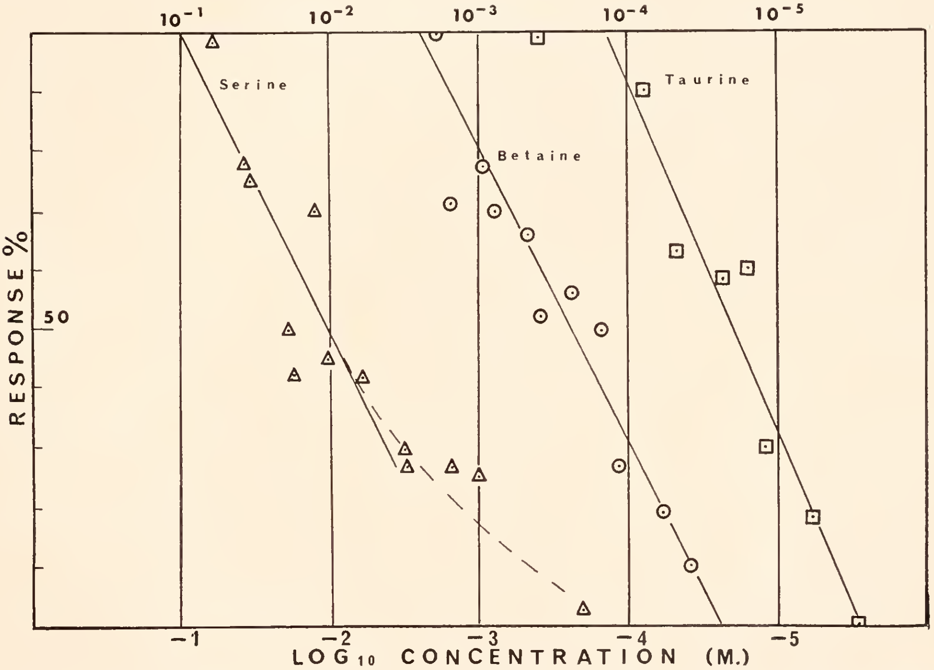

FIGURE 3. Reaction of *Lepas anatifera* to solutions of ampholytes. L-serine: triangles. Betaine: circles. Taurine: squares. The broken line for L-serine indicates predominance of stalk rather than feeding responses in the range of concentration shown (see text).

RESPONSES TO AMINO ACIDS AND RELATED COMPOUNDS

A typical series of results for some amino acids and related compounds, plotted as response against Log concentration, is shown in Figures 2 and 3. The lines are fitted by eye.

The 50% intercepts of these, and of other compounds tested, are listed in Table I. The standard deviation of individual thresholds of response, taken as half the difference in the Log_{10}c values corresponding to 84% and 16%, respectively, of the population responding, are entered in Table I, Column 4. It will be seen that the standard deviations for all the amino acids in which a sufficient range of response could be evoked, and also those for betaine and taurine, varied little about a mean value of 0.6. The threshold concentrations are thus at least four times greater ($10^{0.6}$) in the less sensitive 16 percentile of the population and only one-quarter as large in the more sensitive 16 percentile. In reading the Table it is therefore important to remember that some individuals can respond to concentrations much lower than those listed in Column 3.

TABLE 1

Concentrations of amino acids and related substances required to produce 50% feeding responses by Lepas anatifera

Amino acid and molecular wt.		Median limiting concentration for population response (molar)	Standard deviation** of response (Log_{10} molarity)	Comments
1. Neutral				
Glycine	(75)	2.4×10^{-3}	0.54	
L-Alanine	(90)	3.2×10^{-2}	0.58	
L-Valine	(117)	5×10^{-1}	0.65	
*L-Leucine	(131)	$>1.5 \times 10^{-1}$	—	Weak response
*IsoLeucine	(131)	$>1.7 \times 10^{-1}$	—	No effect observed
L-Proline	(115)	3.2×10^{-4}	0.54	
L-Phenylalanine	(165)	1.4×10^{-2}	0.58	
L-Tryptophan	(224)	$>5 \times 10^{-2}$	—	Weak response
L-Serine	(105)	1.2×10^{-2}	0.70	Stalk response
L-Tyrosine	(181)	2.3×10^{-3}	—	
L-Cysteine	(121)	$>7 \times 10^{-1}$	—	No effect observed
*L-Cysteine	(240)	$>4 \times 10^{-2}$	—	No effect observed
*L-Methionine	(149)	$>2 \times 10^{-2}$	—	No effect observed
L-Asparagine	(132)	$>3 \times 10^{-1}$	—	Stalk response, weak feeding response
2. Acidic				
L-Aspartic acid	(133)	3×10^{-2}	0.60	
L-Glutamic acid	(147)	2.5×10^{-5}	0.68	
3. Basic				
L-Lysine	(146)	4.5×10^{-3}	0.60	
L-Arginine	(174)	3×10^{-3}	0.62	
L-Histidine	(155)	$>6 \times 10^{-2}$		Stalk response, weak feeding response
4. Related Compounds				
Betaine	(117)	2.4×10^{-4}	0.70	
Taurine	(125)	2.0×10^{-5}	0.63	

* Concentration limited by poor solubility.

** The standard deviation given is half the change in Log_{10} concentration, between a 16% and an 84% response by the sample population.

There are great differences in capacity to evoke a feeding response between the compounds listed. L-glutamic acid and taurine are outstandingly stimulating, concentrations of the order of 10^{-5} M being sufficient (Fig. 2). Betaine and L-proline are also very effective, evoking reactions at 10^{-4} M. Four naturally occurring amino acids stimulate at about 10^{-3} M: L-glycine, L-tyrosine, L-lysine and L-arginine; four at 10^{-2} *M:* L-α alanine, L-phenyl alanine, L-serine and L-aspartic acid; five stimulate only weakly at high concentration: L-valine, L-leucine, L-tryptophan, L-histidine and L-asparagine; while L-isoleucine and the sulfur-containing amino acids L-cysteine, L-cystine and L-methionine, are without effect. The stimulating capacity of some amino acids was clearly limited by low solubility, for example, L-valine (Fig. 2). Perhaps isoleucine, cystine and methionine would have had some effect if more concentrated solutions had been possible.

RESPONSES TO ORGANIC CATIONS

Five organic cations were tested, using solutions buffered to pH 8: trimethylamine, trimethylamine oxide, triethylamine, choline, and putrescine. The results are included in Table II. Only choline and trimethylamine gave measurable responses, but the concentration required was greater than that for potassium and ammonium. The response to cations was more uniform than that to amino acids, as can be seen from the values of standard deviation, which approximate to half those for the amino acids.

TABLE II

Concentrations of organic and inorganic cations required to produce 50% feeding response in Lepas anatifera

Cation	Ionic mass	Limiting concentration (molar)	Standard* deviation	Comments
Potassium	39	3.1×10^{-2}	0.31	
Ammonium	18	6.6×10^{-2}	0.38	
Trimethylamine	60	5.4×10^{-2}	0.36	
Triethylamine	102	$>1 \times 10^{-1}$	—	Very slight response
Trimethylamine oxide	76	$>4 \times 10^{-2}$	—	Slight response
Choline	104	1.1×10^{-1}	0.36	
Putrescine	89	$>5 \times 10^{-1}$	—	No response

* See footnote Table 1.

STALK RESPONSES

Three of the amino acids regularly caused slow bending movements of the stalk. When applied at high concentration, L-serine produced an immediate feeding response, usually followed by a stalk reaction a second or two later; sometimes the stalk movements continued for some 5 to 10 seconds and the animal usually remained in the new position it had assumed for much longer periods. When L-serine was applied at concentrations too low to initiate feeding, stalk movements sometimes followed after a few seconds' delay. The response curve for this compound was also anomalous (Fig. 3). L-histidine and L-asparagine among the amino acids gave inconclusive results when tested for feeding reactions, only a proportion of the individuals showing typical responses. Feeding responses were given at a range of concentrations down to about 10^{-2} *M* by histidine but stalk responses, after the usual long delay, occurred down to concentrations of 3×10^{-3} *M*. Histidine could also induce almost closed individuals to open the valves and display the cirri. L-asparagine similarly failed to produce positive and regular feeding responses by the cirri and mouth parts but, like histidine, caused delayed stalk responses when applied to the cirri at concentrations of 10^{-1} *M* which were subliminal for the feeding response. Stalk responses were also noticed when strongly acid or alkaline solutions were applied.

Usually the direction of the movement during a stalk response suggests withdrawal from an unfavorable stimulus; the cirral net moves away from the source of water entering the net (*i.e.* in the sense from rostrum to carina) and weaves slowly from side to side. However, though valve closure would be expected to accompany any reaction to unfavorable stimuli, the valves do not close; in fact, in the case of histidine, they open more widely. The idea of an avoidance response is also inconsistent with the long latency of the reaction, and the fact that it can be produced by concentrations of stimulating substance below those causing feeding responses. Further investigation is required to establish its significance.

Chemoreception in Sessile Barnacles

A few qualitative experiments were made with *Balanus balanoides* and *Elminius modestus* which indicated that feeding activity was stimulated by amino acids at concentrations similar to those used in experiments with *Lepas*. Unfortunately, quantitative methods are more difficult to apply to balanids. They are much more prone to close when shadows are cast on them or when mechanical shocks are applied, and feeding movements are less readily distinguishable from ordinary cirral movements. The turtle barnacle *Platylepas bissexlobata*, like other epizoic forms, is less responsive to shock, and showed very clear feeding responses. It reacted to glutamic acid at concentrations between 10^{-4} and 10^{-5} *M*, and to potassium ions, but not to sugars and other organic substances.

It is reasonable therefore to conclude that the results of experiments on *Lepas* typify the behavior of the majority of cirripedes.

Discussion

The cirri and mouth parts of *Lepas* and probably of the majority of cirripedes are sensitive to two groups of substances: certain of the amino acids and related compounds taurine and betaine on the one hand, and certain cations of the other, notably potassium, choline, and trimethylamine. High concentrations of hydrogen and hydroxyl ions represent conditions unlikely to be experienced in nature, and the animals' response is probably a shock reaction rather than a true feeding response. No responses were elicited by neutral organic molecules, such as alcohols and sugars, nor by anions, whether organic or inorganic, other than glutamate and aspartate.

The two groups of compounds producing feeding reactions in *Lepas* are similar to those that influence feeding and food orientation in many marine carnivores—ctenophores, molluscs, polychaetes, and other Crustacea (Case and Gwilliam, 1963). Electrophysiological studies on higher Crustacea have similarly revealed sensory units receptive to amino acids and tertiary amines. Laverack (1963a, 1963b), using rather high concentrations of trimethylamine (10^{-1} *M*) and other substances containing the group $N(CH_3)_3$, found three types of receptors on the crab dactylopodite which responded to these substances but not to amino acids. Case and Gwilliam (1961) had been able to record from the same preparation units highly sensitive to the L-isomer of glutamic acid and to a lesser degree to some of its derivatives and to other amino acids. Levandowsky and Hodgson (1965) found in preparations of dactylopodite and antennule of *Palinurus argos* evidence for separate units, some sensitive to L-glutamic acid in very low concentration (10^{-4} *M*) and others to tertiary amines (10^{-3} *M*). Hodgson (1958) reported that the antennules and chelae of the fresh-water crustacean *Cambarus* responded well to glutamic acid at very high concentration (0.25 *M*), rather less well to glycine and glutamine, and not at all to a variety of other substances. Similarly, Case, Gwilliam and Hanson (1960), using dactylopodite receptors of the crabs *Libinia emarginata, Callinectes sapidus* and *Carcinides maenas,* obtained positive results for all amino acids tested, with greatest sensitivity to glutamic acid, but no response to alcohols (0.2 *M*), monosaccharides (0.25 *M*), or peptides, including glutathione. The coxal gnathobases, walking legs, and chilaria of the marine arachnid *Limulus* also are sensitive to amino acids but, unlike the Crustacea so far investigated, glycine, not glutamic acid, elicited the strongest response (Barber and Hayes, 1963).

Barnacles therefore resemble other crustaceans in their ability to respond to many amino acids at low concentrations (10^{-2} to 10^{-5} *M*, Table I), and to cations, such as tertiary amines, at rather higher concentrations (10^{-1} to 10^{-2} *M,* Table II). The sensitivity range, as shown by the standard deviation (Tables I and II), was quite different for the two classes of compounds, suggesting different receptor mechanisms as in crabs (Laverack, 1963a, 1963b) and in *Palinurus* (Levandowsky and Hodgson, 1965).

The outstanding and specific sensitivity of the chemoreceptors of *Lepas* and of other Crustacea to low concentrations of L-glutamic acid, a physiologically active compound suspected as a neural transmitter in arthropods (Takeuchi and Takeuchi, 1964), and to substances closely related to neural transmitters, such as betaine and choline, raises the question whether this is a normal gustatory response. Although glutamate was effective at a concentration of only 10^{-5} *M* in the medium external to the sense organ, it is known that concentrations as low as 10^{-10} g./ml. will stimulate *Helix* neurones when directly applied (Kerkut and Walker, 1961). However, not only did glutamate produce a response which resembled normal feeding, but some other substances, not regarded as having a special physiological role, were also able to produce a feeding reaction in *Lepas* at low concentrations, for example L-proline and taurine.

Moreover, although there is a general similarity in the classes of compounds to which marine arthropods respond and a particular sensitivity to L-glutamic acid, the order of sensitivity to different compounds is not always the same for different species, but differs as one might expect of gustatory or olfactory responses. Thus in *Lepas,* proline is the most effective amino acid after glutamic acid, while the peptides ornithine and reduced glutathione are non-stimulatory. On the other hand in *Carcinides,* ornithine has more effect than proline and reduced glutathione also causes a response. Taurine and glutamic acid are the most effective stimuli to *Lepas,* but have only weak effects on the chemoreceptors of *Limulus.* Indeed, if a comparison be made between *Lepas* and *Carcinides,* two crustaceans on which a number of compounds have been tested quantitatively, there is little correlation in the sensitivity ranking of compounds between the two species, and no correlation between sensitivity and any obvious physical or chemical property. There is no general relationship to molecular weight or volume (though peptides and proteins are usually ineffective compared with amino acids), and no relationship to ionization constants or to specific groupings. All that can be said in general is that the most strongly stimulating substances are ampholytes, existing in solution as zwitterions, and that sugars and other neutral substances are wholly ignored.

Some of the substances to which Crustacea are sensitive may afford reliable and efficient guides to the presence of food; scavenging crabs and lobsters would be expected to respond to tertiary amines and betaines present in decaying animal tissue (Laverack, 1963b) as well as to amino acids released by autolysis. But there is no reason to suppose that glutamic acid and taurine, widely distributed as they are (Simpson *et al.* 1959), have any special claim to serve as feeding-inducing substances in the place of other and more commonly occurring amino acids which would also be released. Dr. D. A. Dorsett pointed out to me in this connection that there are many examples in man of strong olfactory and gustatory responses to chemicals which have no adaptive significance. One should not therefore expect that the substances most relevant to the animals' needs will necessarily produce the strongest feeding responses. Crustacean sense organs may just happen to be very sensitive to such substances as glutamic acid but, in suitable circumstances, this high degree of sensitivity might be made to function as a clue to the presence of food.

The responses to amino acids by *Lepas* are at first sight difficult to explain on the basis of food searching and recognition, since the animal is sessile and unlikely often to come into contact with decaying material. However, its food normally consists of living plankton, and there is some evidence that amino acids may diffuse from planktonic animals (Webb and Johannes, 1965). In any case, since the cirri of barnacles are beset with sharp spines, a delicate organism might be pierced on contact with the cirral net, and would very likely be crushed as the flagellum of the cirrus curled around it. If, as a result, stimulating substances were released, a feeding response would reinforce any initial mechanical stimulus, and cause living prey to be devoured. We do not know the composition of the body fluids of planktonic larvae, but if they resemble those of the adults they will often contain amino acids. *Asterias* body fluid evokes a strong feeding response in *Lepas*. Furthermore, although the body fluids may not differ greatly from oceanic water in their concentration of potassium (1×10^{-2} *M*; Dittmar, 1884), the intracellular fluids of animals contain much higher concentrations, often as much as ten times that of sea water (see Prosser and Brown, 1961). Release of potassium sufficient to raise the local concentration to twice that of sea water is evidently sufficient to evoke a feeding response (Table II). The sensitivity of *Lepas* to potassium ions may therefore have some adaptive value in reinforcing the feeding reaction when live prey is caught.

I am indebted to the Marine Biological Laboratory, Woods Hole, for providing facilities and financial assistance for this work, and to Mr. M. Levandowsky and Dr. D. A. Dorsett for useful discussions and suggestions.

SUMMARY

1. Feeding responses were elicited when solutions of amino acids and of organic and inorganic cations were applied to the cirri and mouth parts of *Lepas* and other barnacles. Proteins, peptides, sugars, neutral organic molecules, and anions were without effect.

2. Of the common amino acids, *Lepas anatifera* was most responsive to L-glutamic acid and L-proline. It was very sensitive also to betaine and taurine.

3. The order of sensitivity to amino acids and to other zwitterionic substances did not correlate with any obvious physical or chemical property.

4. Inorganic and organic cations had to be applied at higher concentrations than many amino acids to produce a response and probably acted on different receptors. The potassium ion had the greatest effect of those tested.

5. The observed sensitivity to amino acids and to potassium ions is thought to enable the animal to recognize living prey after it has been pierced by the setae present on the cirri.

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GROWTH AND REPAIR OF SPINES IN THE SEA URCHIN STRONGYLOCENTROTUS PURPURATUS (STIMPSON)[1]

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The following work represents a number of observations concerning the growth and repair of spines on the West Coast purple sea urchin, *Strongylocentrotus purpuratus* (Stimpson). Some of the parts were introductory sorties into a study of natural urchin populations; others were incidental to the main study of urchin ecology and simply suggested themselves as the research progressed.

An examination of the literature concerning the growth of urchin spines suggests that two issues have attracted workers: the presence of growth lines or cycles in the spines which has led to the hope of using them to determine the age of animals; and, secondly, the phenomenon of regeneration of entire spines or spine tips.

Spine sections have been described for many species of echinoids by various authors (Carpenter, 1847, 1870; Mackintosh, 1879, 1883a, 1883b; Křiženecký, 1917; Deutler, 1926; Mortensen, 1928–1951; Swan, 1952; McRae, 1959; Moore, 1966) and the rings, cycles of wedges, or growth lines which appear in cross section have had various interpretations. Carpenter (1847, 1870) and Swan (1952) suggested that these cycles may be formed like the annual growth layers in woody perennial plants. Moore (1966) found a positive correlation between numbers of rings and test volume, and suggested that the rings might be used to determine age in *Heliocidaris erythrogramma*. Deutler (1926) called these rings "growth zones" and thought that they were formed periodically in *Echinus*.

Regeneration of broken spines was apparently first suggested by Quekett (cited by Carpenter, 1870). The first sections of spines showing repair and interpreted as such were made by Carpenter (1870) with *Echinus trigonarius* and *Acrocladia* sp. Borig (1933), using *Echinometra mathaei*, recognized that cycles shown in longitudinal section ended in sharp discontinuities which were the result of breaks and subsequent regeneration. He concluded that cycles were not directly related to regeneration but rather that the spine would not regenerate a new tip until the next "growth period" and that, at this time, cycles would be added even though no break had occurred. The only other reference connecting regeneration with the number of cycles is Swan (1952), who found completely regenerated spines did not have the same number of cycles as original ones. Swan also pointed out that some externally purple spines of *S. purpuratus* had green cores and that regenerated spines were purple, unlike the spines of young animals which were green. The implication was that it should be possible to determine whether a spine was one that had completely regenerated or had grown from the original small spine

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of a young urchin. Part of the puzzle in examining such spines is the sharp distinction between green and purple, which are separated by a cycle of calcite crystals.

MATERIALS AND METHODS

The West Coast purple sea urchin, *Strongylocentrotus purpuratus* (Stimpson) was used in the following studies and all collections were made at the south side of Sunset Bay, Coos County, Oregon, 43° 20′ N. lat. Because various techniques were employed in the different portions of this work, for the sake of continuity, methods will be discussed where appropriate in each section of the results.

RESULTS

A. *Relationship between internal spine morphology and animal size*

At the beginning of this investigation in 1962, it was hoped that cycles present in spines could be used to determine the age of animals. As a first step, the relationship between urchin diameter and number of rings in the spines was examined.

TABLE I

Number of cycles in green-cored spines on urchins collected from Sunset Bay December 8, 1962

Animal number	1	2	3	4	5	6	7	8	9	10	11	12
Test diameter	7.71	6.20	6.20	4.69	4.57	3.51	2.65	2.41	2.41	2.20	2.11	2.09
Number of cycles	9	8	8	6	8	6	6	5	5	5	5	5

Correlation coefficient $r = 0.91$.

In December, 1962, four spines were taken in the field from each of 33 animals. Spines were dried in an oven overnight, dipped in xylene and mounted on microscope slides with "Permount." After hardening, the preparations were ground in longitudinal section. Cycles or rings were counted only in green-cored spines and, when such a spine was found for any animal, no further sections were prepared.

Of the 33 urchins examined, only 12 showed at least one green-cored spine. A positive relationship existed between test diameter and number of cycles (Table I) with a correlation coefficient of 0.91. It was this initial relationship which seemed to promise that age of individuals in years could be obtained from spine morphology, and stimulated further work towards finding the time of year when a cycle would be formed.

B. *A general examination of spine morphology*

Initial efforts to determine the season when new rings were formed was frustrated by lack of a suitable tagging method that would permit individuals in the field to be followed for at least one year. After this problem was solved in the summer of 1963 (Ebert, 1965), 100 animals were marked, a sample of five large primary spines removed from the ambitus of each tagged animal, and the urchins placed in a pool at Sunset Bay. The original plan was to examine the spines and sample more from the same animals at several periods during the year to discover when new cycles were formed.

The handling of spines for preparation of longitudinal sections was modified and eventually approached the techniques of Carpenter (1847) and Deutler (1926) with certain differences. Most of the organic material was removed from the spines with a solution of 5.25% NaOCl (full strength household bleach) as suggested by Swan (1952). If organic material was not removed from the spines, they became brittle when dry and usually fractured during the grinding process. After washing in water for at least three hours and drying, the spines were dipped in xylene, placed on a slide and covered with Canada balsam. They were heated on an electric hot-plate to boil away the xylene, and cooled. When hard, the preparations were ground on a glass plate with #220 followed by #600 carborundum grinding compound. Water was used as the liquid carrier. The slide was tilted during the grinding process to insure the production of a median section. After grinding one side, the slide was returned to the hot-plate, the balsam remelted and the spine turned over, recooled and the grinding completed. The preparation was cleaned in xylene before the coverslip was added. In this manner, 500 sections were prepared.

Figure 1 shows typical sections of these 500 spines. Figure 1A is a cross section showing 10 cycles or rings and is a typical presentation of a so-called polycyclic spine. Each light block (in the outer cycle there are 45) is a single calcite crystal which runs generally parallel with the long axis of the spine. The outside ring of these crystals gives the spine a ribbed or fluted appearance. Between the large crystals are smaller ones which form a meshwork. The dark color is due to the presence of echinochrome.

Figure 1B is a cross section of a spine from the same animal as 1A and shows 4 cycles (52 blocks in the outer cycle). The inner region of the spine is constructed of a loose calcite meshwork. Figures 1C–E are longitudinal sections, are from different animals, and show variations in the arrangements of the cycles of large crystals.

Figure 1C is a portion of a spine tip with the bottom of the picture being towards the base of the spine. The light lines are the large crystals which form the cycles, and the dark regions are fine meshworks of calcite with echinochrome pigment. Distally, the large crystals end abruptly; several fuse towards the base which means, in cross section, that two cycles become one. A major discontinuity is shown towards the bottom of the picture.

Figure 1D again shows large crystals ending abruptly at their distal ends and, towards the tip of the spine, partial fusion of a number of cycles. At the base of the spine, the 3rd, 4th and 5th crystals (starting the count from the outside) all fuse distally to form a single crystal a short distance above the milled ring. The milled ring, a region of muscle attachment, is indicated by an arrow. The fusion of crystals near the base apparently was the nature of the entire cycle because the pattern appears on both sides of the spine. One further important feature is the shape of the large crystals. About in the center of 1D are several bent crystals whose shape must have changed during growth.

In Figure 1E there is a major discontinuity immediately above the milled ring (arrow) which terminates 7 cycles. The outer cycle encloses a fine meshwork of

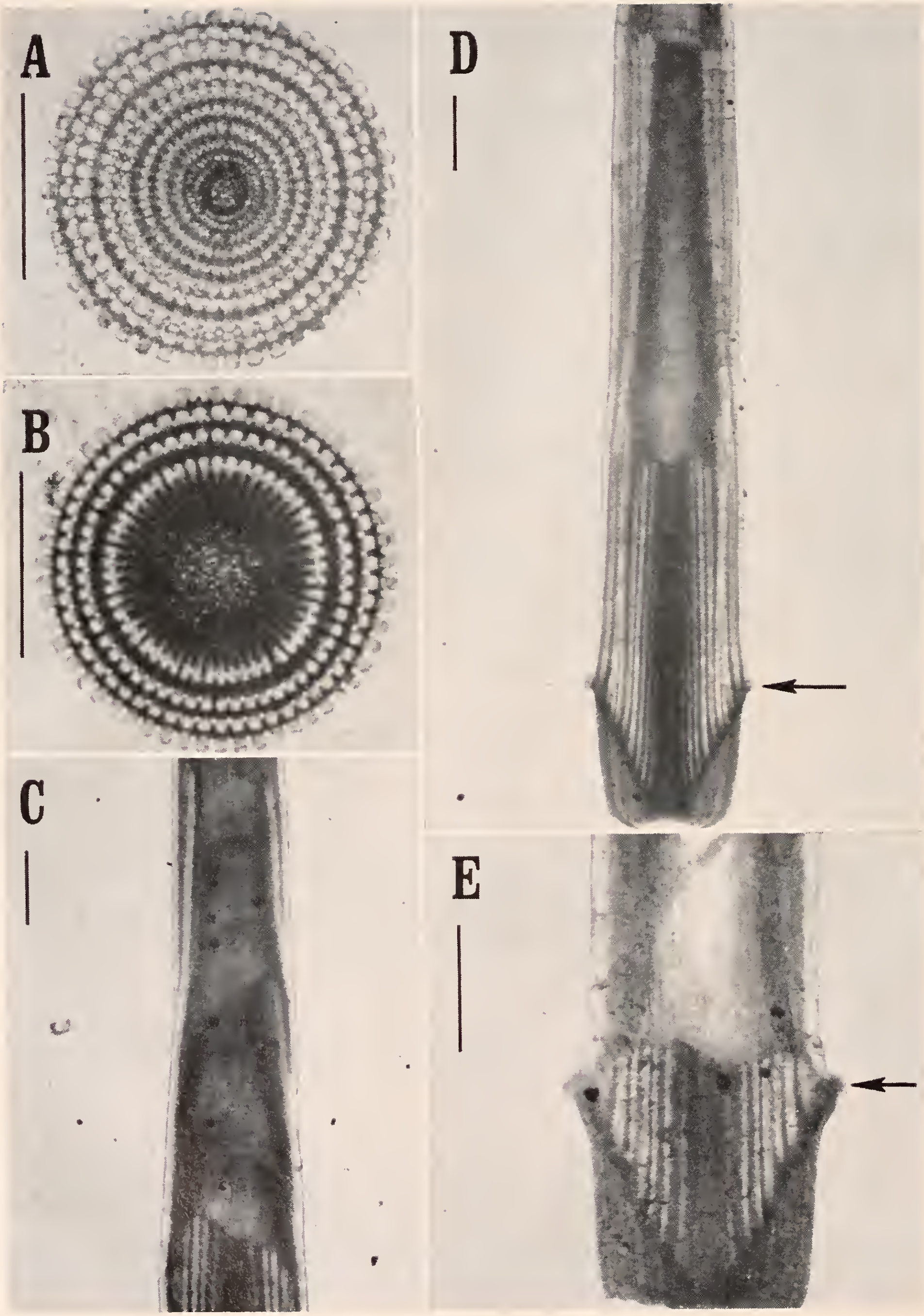

Figure 1.

calcite; however, the central region is coarser in texture and apparently structurally weaker. Spines of this type usually lost this central region during grinding.

Spines have been examined at various times during the year and the morphology of the spines was never different than described above.

C. *Formation of discontinuities between green and purple in spines*

On October 2, 1963, urchins of age class 0, *ca.* 1 cm. in test diameter, were observed at Sunset Bay. One group of 40 animals in a small tidepool was completely covered with debris consisting of shells, shell fragments and pieces of algae. All of these animals were green. A second group, also of age class 0 and consisting of several hundred individuals, was living in a large tidepool and not covered with debris. All of these were purple. A possible explanation was that the production of echinochrome was, in some fashion, related to light. In early marking experiments, there was an indication that pigment production was also related to injury or that echinochrome was moved to areas of irritation. A simple experiment was designed to investigate both light and injury. Two 1-gallon glass jars were filled $\frac{3}{4}$ full of sea water and placed in an 11° C. constant temperature room. Eight age class 0 urchins were prepared for each jar: four were green and the other half purple. Spine tips on two green and two purple animals of each group were clipped. The animals were photographed and placed in the jars which were then sealed. A small hole was allowed in each lid to permit the passage of a rubber hose for an air supply. Aluminum foil was wrapped around one jar, occluding all light, and a 100-watt lamp was placed one foot away from the other. The experiment was continued for one month. Animals were again photographed on November 21 and December 2, at which times the urchins in the darkened container were, of course, briefly exposed to light.

Results of spine growth after one month under conditions of darkness showed regenerating spine tips were white to greenish on both green and purple urchins. Under light conditions, regenerating spine tips were purple in both green and purple urchins. The first conclusion is that light is important in echinochrome synthesis or transport and, secondly, that a sharp discontinuity can arise between green and purple in young spines through breakage and regeneration under light conditions. This would mean that the sharp line at the top of a green core represents a break and regeneration of a small urchin's spine under light conditions.

D. *Relationship between regeneration and internal spine morphology*

A further examination of the role of regeneration in determining the distribution of cycles in spines was started on May 4, 1964, when a sample of urchins was collected and brought back to the University of Oregon, placed in aquaria of aerated sea water and kept at 11° C. Four animals (4.15–7.15 cm. test diameter) were individually marked with nylon monofilament through an ambulacrum (Ebert, 1965) and returned to the aquaria. Tips of all primary spines in the interambulacrum next to the mark were removed and placed on file cards in the order of

FIGURE 1. Ground sections of *S. purpuratus* spines. A and B are cross sections taken above the milled ring; C–E are longitudinal sections; the milled ring is indicated by an arrow. The scale lines are 1 mm. Black spots are particles of grinding compound.

removal on May 8. The position of the mark on each animal was mapped to insure proper matching of the tips with the spines at a later date. Spines were collected again on July 8, 1964, cleaned with NaOCl, matched with their previous tips, mounted on slides and ground in longitudinal section so that the cycles could be examined.

Figure 2 shows a typical pairing of a spine tip with its original spine after two months of regeneration. A new cycle of calcite crystals formed during this time. The break shows as a discontinuity in the regenerated spine similar to the sharp lines seen in the spines in Figure 1.

DISCUSSION

Discussions by Mortensen (1928–1951) and Swan (1952) during the initial stages of this work, to a great extent, were responsible for the original hope of being able to determine age of animals by examining spine morphology. A further consideration which strengthened this belief was the positive correlation between size of animal and number of cycles in green-cored spines. After examining a large number of spines during 1963, I was led to form an alternate hypothesis for the formation of cycles, *viz.* that they were formed when a spine tip was broken and regenerated. This was proposed because: (a) spines always had a cycle of crystals on the outside. If the cycles were formed only at certain periods during the year, then, at some time, the fine crystalline meshwork would have to appear on the outside; (b) in longitudinal section, the cycles were always distally terminated at a sharp discontinuity which suggested a break. The two experiments with regeneration in the laboratory, production of the sharp color discontinuity in spines of small animals and the addition of a new cycle of calcite crystals in larger animals, conclusively showed that the alternate hypothesis was correct and that cycles have no direct relationship with age other than the very simple one, that breaks tend to accumulate in a spine through time. Old animals, as a result, would tend to have more cycles in their spines than would young animals. This is the proper interpretation of the positive correlation between number of cycles and size. Spines that have been completely regenerated would show fewer cycles and no green core, as suggested by Swan (1952).

Although no work has been done on the histology of the spines during growth and regeneration, some ideas can be presented concerning possible occurrences which would be consistent with observed spine sections. Following a break, the dermis on the remaining spine stump must be sloughed off or altered in some fashion so that when regeneration begins, the former outer ring of large crystals is simply covered over by new growth. A new dermis probably is formed of cells originating from within the existing spine shaft rather than growing up from the milled ring, because regeneration appears to begin on the broken stump. That the dermis is injured in some fashion seems consistent with the partial regeneration lines seen in Figure 1C and D. Partial regeneration would result if damage was not great enough to affect the dermis of the entire spine. At the top of Figure 1D, a series of four regeneration lines merge into a single line. The arrangement is best interpreted as multiple regeneration where a small break occurred and was repaired (the innermost cycle at the top of the spine). Growth of a new tip took place with new outer crystals merging with original outer crystals. The tip

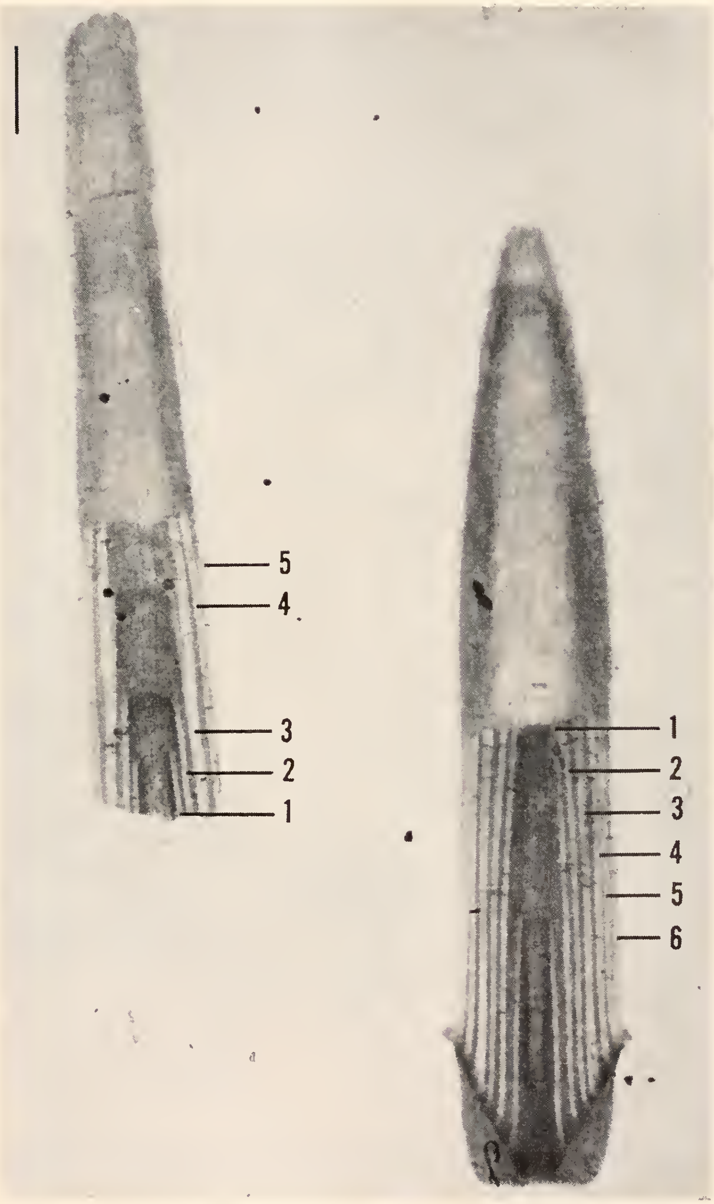

FIGURE 2. Matched tip and regenerated spine. The cycles are numbered and indicate that during regeneration of a new tip, a new cycle was added (6). The scale line is 1 mm. Black spots are particles of grinding compound.

was broken again at the same point as the first break. The extent of influence of the break apparently was greater, and the next set of calcite crystals was added further down the shaft of the spine. This sequence was repeated until the effect of breakage was enough to cause a cycle to be added all the way to the milled ring. That a spine would break at the same place a number of times is not too unlikely because new material formed at the site of a break is apparently weaker than that deposited later. Mackintosh (1879) suggested that the hollow appearance of certain spines was associated with repair. Carpenter (1847, 1870) also felt that there was a relationship between large regions of regeneration and formation of structurally weak calcite meshworks. Such weakened zones, as shown in Figure 1E, would be likely sites for multiple breaks.

Křiženecký (1916) suggested that when a spine was broken near the tubercle, it was shed and an entire new spine was formed. This seemed to be generally true in this study; however, as Figure 1E indicates, a break can occur almost to the milled ring and the spine still regenerate. It is possible that the critical factor for determining spine loss after breakage may not be the absolute amount of material lost but rather the damage done to underlying tissues such as the inner and outer muscle layers attached to the base of the spine. The fusion of calcite crystals in Figure 1D near the milled ring may have resulted from damage to the supporting tissues of the spine. In such a case, damage would not have been great enough to cause spine loss.

It now seems possible that ideas concerning the growth of echinoid spines must be modified. Spines may not grow simply by addition of new material to the outside. Large calcite crystals forming a cycle are always on the outside of the spine, yet the spine increases in size and a fine mesh of calcite develops between the outside cycle of large crystals and the next inner cycle. Furthermore, calcite crystals, if bent, may be straightened during growth, as suggested by Figure 1D. All calcite parts are bound together in a three-dimensional lattice, and so, to maintain the observed arrangement, calcite must be resorbed as well as deposited. Resorption of test plates has been discussed by Lovén (1892), Jackson (1912), Deutler (1926), Gordon (1926), and Mortensen (1927), and was experimentally shown by Cutress (1965). The highly precise and dynamic nature of growth, however, was not indicated by previous workers. Growth of spines, as shown in this study, may be as complex as vertebrate bone growth with its interaction of osteoblasts and osteoclasts. Ordered resorption and deposition in other invertebrates have been indicated in brachiopods (Hyman, 1959; p. 529) and, to an apparently lesser extent, in molluscs (Wilbur, 1964).

SUMMARY

1. Numbers of cycles in the spines of *S. purpuratus* were positively correlated with animal size.

2. Examination of 500 spine sections in this study led to the hypothesis that cycles are the result of breakage and regeneration.

3. The relationship between breakage, regeneration and internal spine morphology was established by producing sharp lines of color in the spines of young urchins and new cycles in the spines of larger animals, both as a result of regeneration.

4. From observed morphology and experimental evidence, it appears that growth of spines requires a very precise resorption as well as deposition of calcite.

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SHORT-TERM PHOTIC REGULATION OF A RECEPTOR MECHANISM IN A DINOFLAGELLATE[1]

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The directional response of organisms with respect to a light stimulus has been under investigation for over a century. Study of the phototaxis of unicellular organisms was initiated by Cohn (1865), who noted that many microörganisms respond to blue light by a change in swimming direction with a subsequent orientation toward the light source. However, in spite of the voluminous literature on the subject now at hand (*Vide* Haupt, 1959; Bendix, 1960; Halldal, 1964; Jahn and Bovee, 1967), advances in the understanding of the mechanisms by which this process takes place are still few and unrelated. There exists great variability in response from one form to the next, and even within a given species. Several investigators have noted extremes of responsiveness, depending upon the age of the culture (Halldal, 1956), the time of day (Bruce and Pittendrigh, 1956), and even such factors as ion balance and pH (Halldal, 1959).

It is obvious that the phototactic process involves much more than a simple light-to-photoreceptor-to-"motorium" relationship. The regulating mechanisms involved must be elucidated and their relationship to the photoactive machinery determined if the consequence of phototaxis with respect to the ecology of the organism is to be understood.

It is the purpose of this report to give evidence for a photoregulatory system and its possible relation to the receptor-effector chain in the phototactic response of the dinoflagellate *Gyrodinium dorsum* Kofoid.

Material and Methods

Culture methods

Cultures of *Gyrodinium* were grown in a sea-water-based medium as previously described (Hand, Collard and Davenport, 1965). The cultures were maintained in a light- and temperature-controlled environment, the light being furnished from above by eight 20W daylight fluorescent tubes and a 60W incandescent lamp. The total illumination at the surface of the culture was approximately 400 foot-candles. The light was cycled to give a 16–8 light-dark daily period. Temperature in the culture box was maintained at 20° C. Under these conditions cultures came to peak growth (approximately 10,000 cells/cc.) in two weeks. Cultures from 4 to 5 days old were selected for experimentation because of their high degree of responsiveness; cultures one week or more old were found to give a generally poor light response. All experiments were conducted between the fourth and sixth hour of the light phase (9–11 AM). Room temperature was maintained at 20° C.

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Apparatus

Experiments were conducted using the flying-spot scanning apparatus previously described (Davenport, Wright and Causley, 1962; Hand *et al.*, 1965). Its operation may be briefly described as follows: An oscilloscope tube generates a square sweep of low intensity light. This scan passes through the sample and is perceived by a photo-multiplier tube. An organism intercepting the scanning beam is reported as a potential change by the photocell. This potential change is ultimately displayed on a viewing readout oscilloscope as an image of the organism, giving its size and position. In effect, the image seen is the "shadow" cast by the organism.

By coupling a Dumont type 450 oscilloscope camera fitted with a Robot "Recorder 24" 35mm. camera-back to the display scope, the images of moving organisms may be photographically recorded on Tri-X Pan film as they occur. Hence, in a single picture of the display screen, one can obtain data from a number of organisms for linear velocity, rate of change of direction, aggregation, and orientation (the type of data gathered being dependent on scan rate and film exposure). In some experiments, where a running sequence of events was desired, a Bolex H16 Rex III motion picture camera was used to collect the data.

For the experiments to be described, the following additions were made to the basic apparatus. The organisms being scanned were stimulated by light from a Bausch and Lomb No. 33-86-02 Grating Monochromator. This instrument was adjusted to give a 20 mμ band-pass between 400 and 700 mμ. Intensity was controlled with an Optical Coating Laboratory continuously graded neutral density filter. Stimulus duration and interval were controlled by a simple revolving blanking wheel in which a notch of the required size was cut. The wheel was driven by a Bodine type NSH 128 motor. Wheel velocity was controlled by a rheostat. A stimulus marker was operated in conjunction with the revolving disc.

In some experiments, a second red light source was used. Its beam entered the preparation under study at a 90° angle to the axis of the stimulus beam from the monochromator. The light source consisted of a Toyoda microscope lamp. Its intensity was controlled by the associated Toyoda rheostat. The wavelengths of this source were controlled with a Wratten A No. 25 glass filter, having a low spectral cut-off at approximately 580 mμ. This filter allowed essentially all the energy in the light source between 600 and 700 mμ to pass.

Because the Toyoda lamp produced a very hot emission, a "heat sink" was introduced between the light source and the sample. This consisted of a 20-cm. Plexiglas tube filled with distilled water. Under these conditions the red light produces only a 1.0° C. temperature change after 30 minutes illumination at the scanning site.

Responses and data collection

Two responses were studied: the "positive" phototactic or migratory response and what we have called the "stop-response." Under the conditions of our experiments a "negative" phototaxis has never been observed. The stop-response has been observed in many forms and was given the name "shock reaction" by Engelmann (1879). It consists of a rapid cessation of movement following a brief but intense light stimulus. As will be seen, this response provides an excellent tool for the study of the photoreceptor machinery, because of its all-or-none nature.

For the investigation of responses cells were placed on the microscope stage in a specially designed microscope slide (Fig. 1), in which the total cell preparation could be subjected to light from one or both sources.

Data recording was accomplished by photographing the appropriate response. The method of data collection depended on the kind of experiment being conducted, and consisted of counts of cells, comparisons of numbers of cells moving with numbers of cells not moving, and orientation of the cells with respect to a given light source. In experiments where a motion picture camera was used, a frame-by-frame analysis of the film was performed to obtain the data.

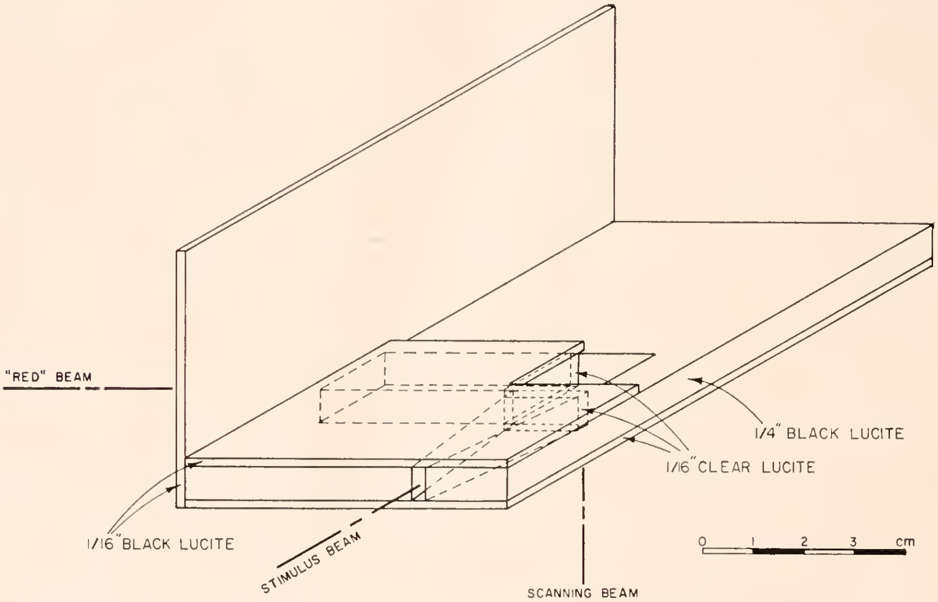

FIGURE 1. The preparation slide. The cell suspension is contained in the center well. The axes of the light beams intersect in the center well through clear Plexiglas windows.

EXPERIMENTS

I. *The action spectrum of phototaxis*

Positive phototaxis in *Gyrodinium* consists of a migration of cells toward the stimulus source, resulting eventually in the accumulation of individuals at the wall of the slide-well through which the stimulus light enters the well. This area is scanned, and a photograph is taken at the time the stimulus is introduced and again after the cells have been illuminated for one minute. The total number of cells in each case may be counted and compared, with the resulting ratio giving the indication of phototactic activity. As a control a series of tests was run in which no stimulus was given. In this series the possible effects of raster illumination or of irregular distribution by the initial pipetting were eliminated.

For a single-beam action spectrum determination of this kind to be correct, wavelengths must be of equal energy. To accomplish the energy equilibration, the monochromator was calibrated using an Eppley vacuum thermopile. Readout from the thermopile was taken with a Keithley Model 149 Mill-Microvolt Meter. The light from the monochromator was passed through the neutral density filter and a plate of Lucite before being intercepted by the thermopile. This was done to insure that the resulting energy measurement was equal to that which the cells would encounter under experimental conditions. The initial calibration adjusted all energies from 420 mμ through 550 mμ to the maximum energy at 420 mμ. A phototactic response curve was determined at this energy in a point-by-point manner. A 0.6-ml. aliquot was taken from the culture, and placed in the slide well. The resulting preparation was covered with a standard glass coverslip, removed from the culture chamber and placed on the scanning stage in the dark. It was allowed to stand undisturbed for three minutes before being tested for the response, this time being necessary to allow the cells to reach random distribution in the well. The sample was then stimulated in the prescribed manner and following photography was discarded. Several samples were taken at each wavelength to insure an adequate sample size (n = 100). The resulting action spectrum may be seen in Figure 2.

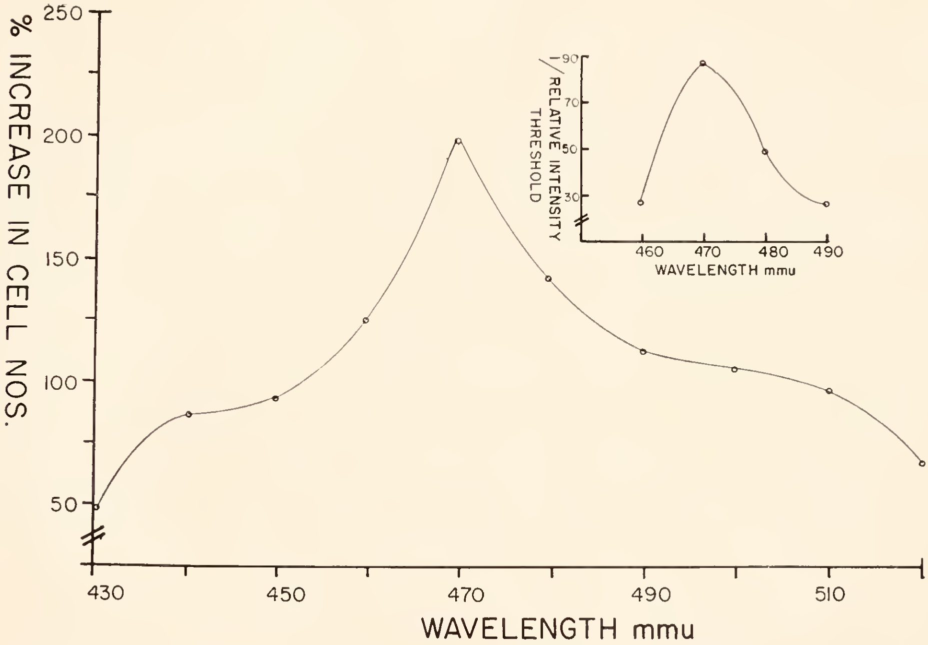

FIGURE 2. The action spectrum for positive phototaxis as determined by an equal-energies method. The points determining the curve represent the maximum increase in cell numbers (accumulation) following a given stimulus. Inset: The action spectrum for the active spectral region of positive phototaxis. The points determining the curve represent the lowest relative intensity at which maximum orientation was observed during a 10-second stimulation.

In order to determine as accurately as possible the "peak area" of this spectrum, the region between 460 and 490 mμ was re-examined using a threshold determination method. The intensity threshold was defined in this series of experiments as the intensity which elicited maximum orientation during a 10-second illumination. The prepared sample was allowed to remain in darkness for 3 minutes, then a 10-second light stimulus of a given intensity and wavelength was administered. A 2-second camera exposure recorded the orientation of the cells with respect to the stimulus direction. This process was repeated, each time with a new sample, until an intensity was reached in which no significant orientation could be observed. Cell pathways exhibiting orientation within 10° of the axis of the stimulus source were compared to the total number of cells present. The resulting ratio indicated the cell sensitivity to the tested wavelength and intensity. The threshold values were corrected for the initial energy differences present in the stimulus source and plotted (Figure 2, inset).

The action spectrum determined for phototaxis in *Gyrodinium* indicates that a single peak for orientation and migration exists at 470 mμ. Because of the wide band-pass of the monochromator we must add an error factor of ± 10 mμ to this determination. A peak around 470 mμ is consistent with the findings of other investigators for dinoflagellates. Halldal (1956) reported that the dinoflagellates *Gonyaulax polyedra* and *Peridinium trochoideum* gave a peak phototactic response at 475 ± 7 mμ.

II. *Some characteristics of the stop-response*

Motion pictures (16 f.p.s.) were made of the stop-response resulting from a 470 mμ light stimulus of 10 seconds duration. Each frame was projected on to a paper screen. The position of each cell in the frame was marked, and the next frame projected and the cells marked. The resulting sets of marks formed the pathway of the cells just prior to and during the stimulus period.

A response latency was calculated, based on the number of position changes following the initiation of the stimulus. These calculations yielded a response *latency* of 400 to 600 msec. Furthermore, it was determined that the response *duration* was 4 to 6 seconds.

In any sample of these cells, a certain amount of stopping occurs without external stimulation. This response is most often caused by encounters between cells, but can occur without any visible external factors. We term this response "random stopping," and in the process of running other experiments, we have found that it can occur in as many as 30% of the cells present during a given period of time. An average value for a sample size of 100 individuals taken from an early (5-day) culture is 14%.

The motion pictures were also examined for the direction of movement taken by the cells following the stop-response. In 50 cells examined, 39 migrated directly toward the stimulus source. The remainder of the cells chose more devious routes, but eventually exhibited a positive phototactic response.

The pathways of cells which failed to stop during a light stimulus were also examined. It was found that these cells failed to respond to the stimulus in any way during the stimulus exposure.

III. *The action spectrum of the stop-response*

If the stop-response is indeed the result of the same photo-machinery responsible for the phototaxis, then both phenomena should have similar characteristics with respect to wavelength sensitivities. To test this, an action spectrum determination was conducted on the stop-response and compared to that previously obtained for the phototactic response.

The action spectrum for the stop-response was also obtained using a threshold determination method. Following a one-minute equilibrating period in the dark, the prepared sample was given a stimulus of 2 seconds at the maximum intensity obtainable for that wavelength. The response was recorded using the still camera at a ¼-second exposure, one second after the initiation of the stimulus. Following stimulation, the cells were allowed to remain in darkness for 20 seconds; then they were again stimulated at a lower intensity. This process was repeated until an intensity was reached at which no stop-response above random could be recorded. The wavelength band examined was between 430 and 530 mμ. As in the orientation experiments, the samples were run at 10 mμ intervals, and enough samples were tested to record the responses of at least 100 individuals.

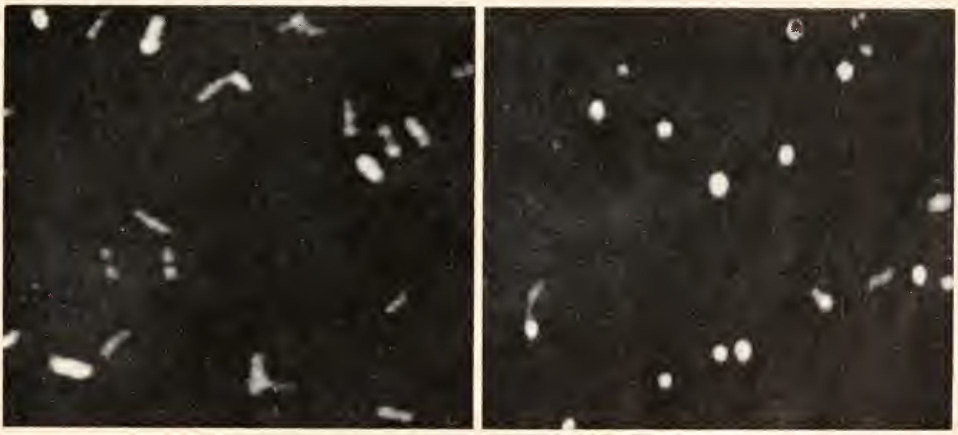

FIGURE 3. One-quarter-second exposures. Left: cells in motion. Right: stop response.

"Threshold" was defined as the intensity at which 50% stopping was obtained. The number of cells stopping was compared to the total number of cells present in the sample to obtain the per cent stopping value. Stopped cells appear on the film as dark circular to ovoid spots, while cells moving appear as less dense rod-shaped marks. This is shown in Figure 3. The values resulting from these counts were corrected with respect to the original monochromator energies and plotted. The resulting action spectrum curve is given in Figure 4.

In examining the action spectrum of the stop-response, it is apparent that the peak coincides with that obtained for phototaxis. On the basis of these results it would appear that the same pigment system operates in both responses. This assumption is not unjustified if one considers the findings concerning the direction of cell movement following the stop-response reported in Experiment II.

As can be seen by comparing the curves for the two responses, the phototactic response threshold is much lower than that obtained for the stop-response. This threshold difference gives some indication as to why the stop-response is generally followed by a migratory response.

IV. *Dark-adaptation of the stop-response*

If the photoreceptor of this organism is related in function to that of an animal photoreceptor, then it should show similar properties with respect to irritability. One quantitative relation between photic stimulation and response magnitude is described by the reversible reaction of light- and dark-adaptation. If a receptor is presented with high intensity stimulation for an extended period, the sensitivity of the receptor falls. If a receptor in this "light-adapted" state is put into darkness, and its sensitivity monitored, the characteristic dark-adaptation curve may be recorded. This curve represents a rise in sensitivity presumably due to the regeneration of a photoactive pigment. In many photoreceptor systems such a curve has been demonstrated. Where the curve involves only a single pigment it has a characteristic monophasic shape.

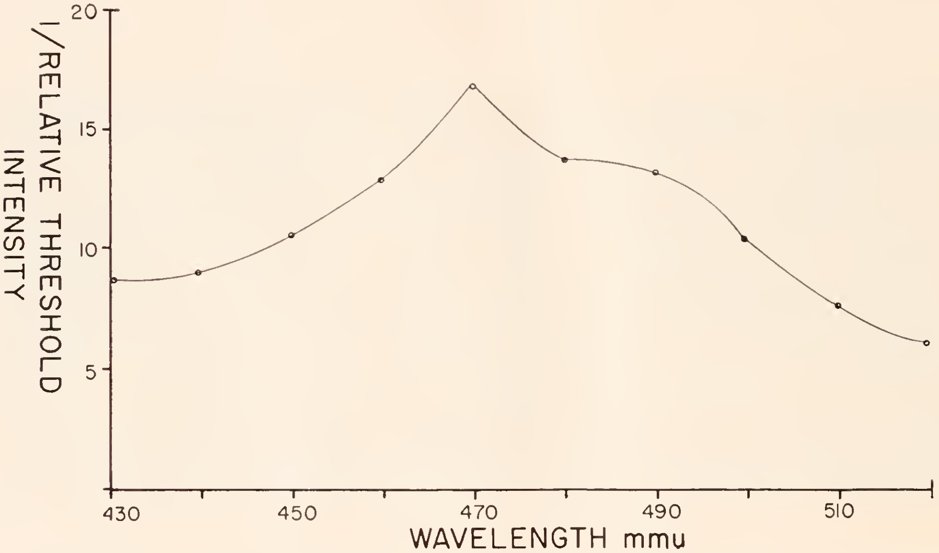

FIGURE 4. The action spectrum of the stop-response as determined by a wavelength-threshold method. The points determining this curve represent the relative intensity per wavelength at which 50% stopping was obtained (n = > 100).

In the experiments described below, the dark-adaptation curve for the stop-response is determined.

Light-adaptation was accomplished by bathing the cells in a 1000-foot-candle tungsten white light for 5 minutes. Following this the cells were put in darkness and stimulated at a given intensity for 2 seconds at 15-second intervals until the cells returned to maximum sensitivity. The response to each stimulus was recorded with the still camera using a ¼-second exposure, given one second after the initiation of the stimulus. The sample was discarded, a new one prepared, light-adapted as before, and then placed in darkness and stimulated at a lower intensity. This process was repeated until the stimulus intensity administered would not elicit any response in 10 minutes. Several samples were taken at each stimulus level. A second series of experiments was performed in which the light-adaptation time was extended to 10 minutes. The results of both experiments may be seen in Figure 5. As the results indicate, the 10-minute light-adapting time gave the same results as the 5-minute time. This indicates that 5 minutes at this intensity is sufficient to "bleach" the photoreceptor pigment completely.

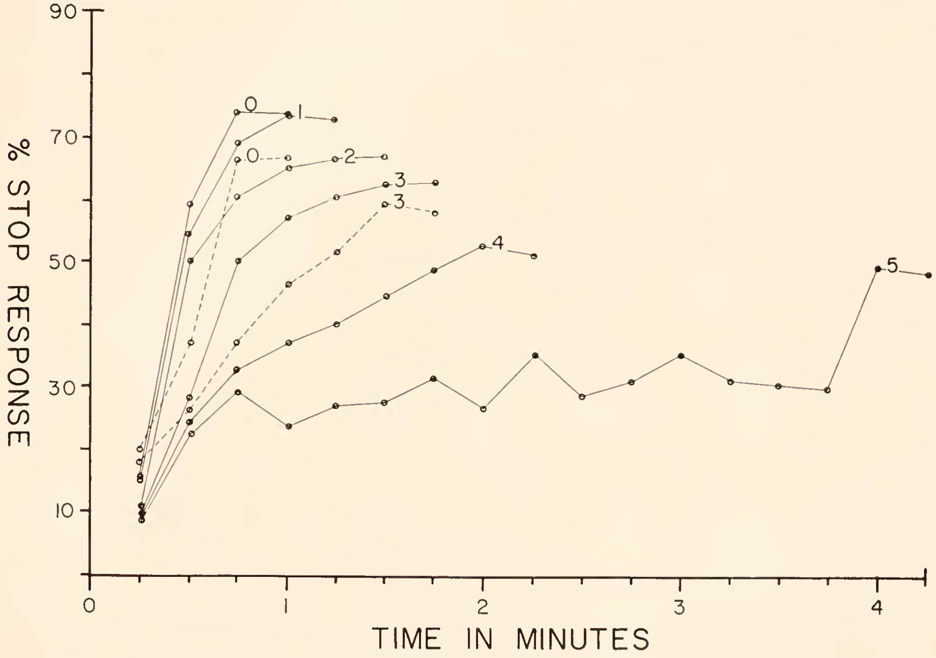

Figure 5. The relation of stimulus intensity to stop-response magnitude when light-adapted cells are placed in darkness. The points represent the return to maximum response shown by 100 cells, when these cells are stimulated at different intensities. The numbers represent the different stimulus intensity levels, with 0 = 280 ft.-c., 1 = 213 ft.-c., 2 = 170 ft.-c., 3 = 136 ft.-c., 4 = 113 ft.-c., 5 = 89 ft.-c.

The dark-adaptation curve (Fig. 6, I) was derived from Figure 5. The points determining the curve represent the time at which maximum response was reached at that particular stimulus intensity. It may be argued that the points best representing a threshold value are those points at which the maximum increase in response occurred at the various test intensities. Yet it must be emphasized that the points making up this distribution are based on a population value, and not on any individual. Hence, the maximum response value, as chosen, most closely represents the time in which the largest number of cells in the population regain sensitivity to a given stimulus.

As can be seen, the dark-adaptation curve determined for this behavioral response of *Gyrodinium* resembles in form that obtained by Hartline and McDonald (1947) for single isolated photoreceptors of the horseshoe crab, *Limulus*. This resemblance leads us to believe that the response we are measuring in *Gyrodinium* does directly reflect the nature of photoreceptor action, and that this action involves a regenerative pigment.

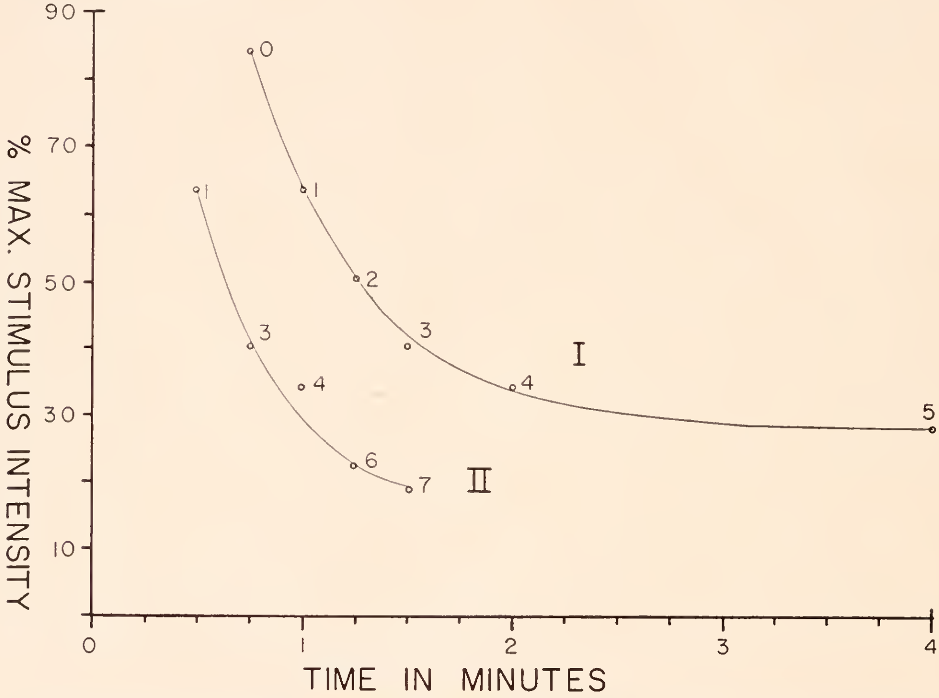

FIGURE 6. (I) The dark-adaptation curve for *Gyrodinium*. The points determining the curve represent the time at which the maximum number of cells responded to each (numbered) stimulus level. (II) The dark-adaptation curve for *Gyrodinium*, when red light is substituted for darkness following light-adaptation.

V. *The effects of darkness and red light on the stop-response*

In early work with *Gyrodinium* we observed that the cells, when placed in darkness for an extended period of time, did not retain their ability to give an orientation or stop-response but did maintain normal motility. A series of experiments were designed to determine the length of time the cells could give a maximum stop-response after being placed in the dark. Samples were placed in darkness and stimulated for 2 seconds at one-minute intervals, using maximum energy at 470 mμ as the stimulus. As a control against the possibility that repetitive stimulation adapted out the response, a second sample was tested in which the cells were stimulated at time 0 and at time 10 minutes. The resulting curve is shown in Figure 7. As can be seen, the response diminishes to one-half of its original value in 10 minutes. The response maintains a high normal value for approximately 6 minutes before degrading. This fact became most important in the design of all experiments described in this paper, in that the total time of testing was kept at or below 5 minutes.

When experiments were begun to test the wavelength sensitivity of the photoreceptor system, it was observed that red light (betwen 600 and 700 mμ) had a profound effect on the response decay phenomenon, but did not elicit any overt cell response. The effect of red light was examined by placing the cells in red light rather than in darkness and stimulating them at one-minute intervals as before. There was extreme variability in the total time the stop-response could be maintained from sample to sample, but it was determined in all cases that cells would maintain maximum sensitivity to the blue stimulus for 15 minutes.

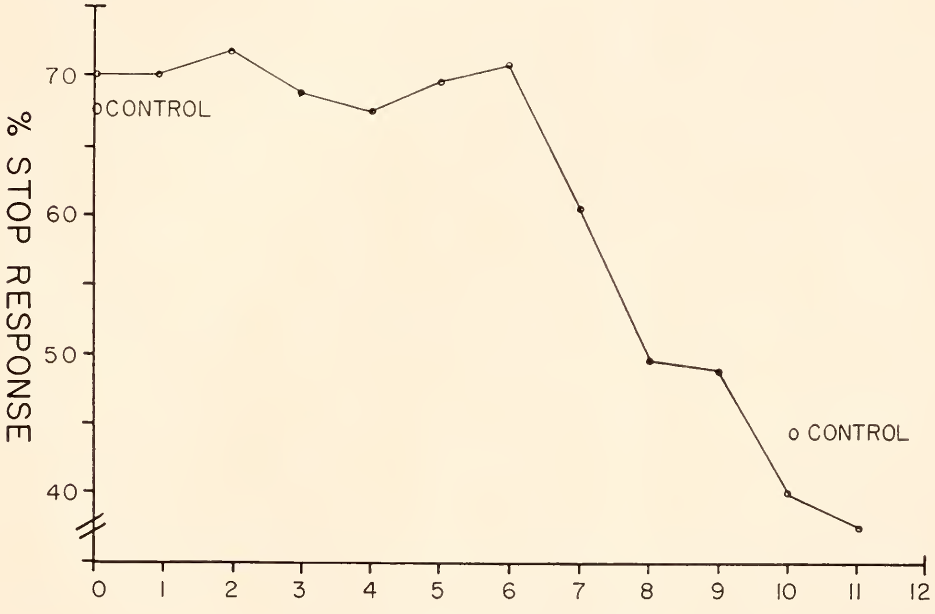

FIGURE 7. The loss of stop-response when cells (n = 100) are placed in darkness. The stimulus is a 280 ft.-c. 470 mμ light of 2 seconds duration, given every minute. The controls represent a stimulus given at 10-minute intervals.

A series of experiments was then designed to define more clearly the relationship between the red- and blue-absorbing pigment systems. The cells were subjected to given combinations of red light, blue light, and darkness, and stimulated with maximum intensity 470 mμ light (280 ft.-c.) for 2 seconds at five-minute intervals. The total time of the experiment was 15 minutes. In cases where blue light was to bathe the cells, this light was maximum intensity 470 mμ light from the monochromator. To effect a true stimulus with this source, the bathing light was interrupted for 15 seconds, thereby placing the cells in darkness briefly before the next stimulus was administered.

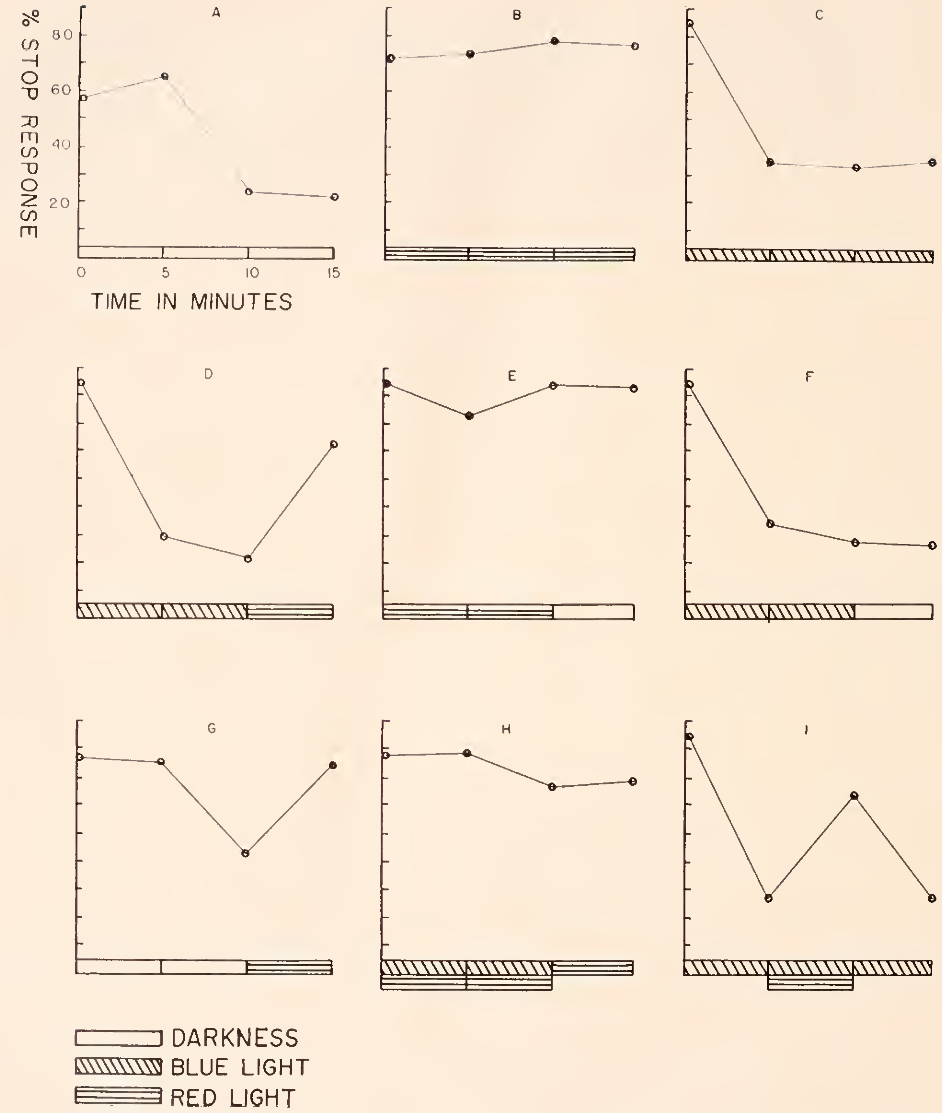

FIGURE 8. The effect of various combinations of red light (600 to 700 mμ at 300 and 280 ft.-c.), blue light (470 mμ at 280 ft.-c., except for 8, F which is 440 mμ at 240 ft.-c.), and darkness on maintenance and regeneration of the stop-response. Each point represents the responses of > 100 individuals.

These experiments were first conducted with the energies of the blue and red sources equal. The series was repeated with unequal intensities, the red being the higher (300 ft.-c.). The results were the same as those of the equal energy series. The combinations of light used in these experiments and the results are shown in Figure 8.

These experiments clearly indicate that red light has a direct effect on the maintenance of the sensitivity of the blue-absorbing system. Cells placed in darkness lose sensitivity over the 15-minute test period (Fig. 8, A), as would be expected, while cells placed in red light during the same test period maintain full sensitivity to stimulation (Fig. 8, B). Cells placed in blue light, with a spectral peak of 470 mμ, lose their sensitivity within the first five minutes rapidly, this presumably being due to light-adaptation (Fig. 8, C).

It is quite possible that photosynthesis is responsible for the "red effect." If so, then blue light of the proper wavelength (440 mμ, 240 ft.-c) should have some maintaining function for the receptor response. To test this, such light was administered for 10 minutes, followed by a 5-minute dark period, this dark period being given to allow the photoreceptor to dark-adapt (Fig. 8, F). As the results indicate, an effect of blue light to maintain the response could not be demonstrated. A similar experiment was performed using red light of the same intensity for 10 minutes, followed by darkness for 5. In this case the response was maintained (Fig. 8, E). This set of experiments does not by any means exclude the possibility that the red effect is due to photosynthesis. It is quite possible that the intensity required for blue light to maintain the stop-response is much higher than that required by red light. Most recently, Diehn and Tollin (1966) have presented some information which suggests that in *Euglena* photosynthesis may be involved in the maintenance of the phototactic process.

In order to determine whether the red system can regenerate the stop-response once it has disappeared, the cells were placed in darkness or blue light for 10 minutes, followed by 5 minutes of red light. As Figures 8, G and 8, D show, the response declined during the first 10 minutes but returned after the cells were in red light for 5 minutes.

Finally we wanted to know what would happen if the cells were given both red and blue light simultaneously. Both light bands were given for 10 minutes, then only red for the last 5. We expected evidence of light-adaptation to blue light by the receptor, as indicated by a low response during the first 10 minutes. Unexpectedly, the response was maintained at the normal high level throughout this time (Fig. 8, H). On the basis of these results we feel that the red process is capable of regenerating the blue photoreceptive system at a rate equal to or faster than the pigment is inactivated at this stimulus intensity. This can be more clearly seen in the last experiment (Fig. 8, I). Here the cells were placed in blue light for the full 15 minutes, but during the second 5 minutes red light was added. As the results indicate, the response level rose, indicating a regeneration of the blue-absorbing system even while the cells were in the presence of constant blue illumination.

VI. *Dark-adaptation in red light*

The dark-adaptation experiment was repeated taking into consideration the effect of red light on the blue photoreceptor. The procedure was the same as that previously described with one change. The sample was placed in red light rather than in darkness following light-adaptation and tested as before. The results of these experiments are shown in Figures 9 and 6, II. The cells dark-adapted faster in red light and also attained a lower threshold.

If one compares the maximum response level attained at each stimulus intensity in darkness (Fig. 5) with similar data obtained in red light (Fig. 9), one sees a decline in the maximum response level in the former. This difference may be attributable to the fact that under the former condition the blue-absorbing photopigment is rapidly bleached by successive stimulation and in the absence of red light cannot be reconstituted rapidly enough to maintain a high response level. In the latter, red light is present, and as has already been demonstrated, the presence of such light allows for regeneration of the blue-absorbing pigment at a rate faster than the sucessive stimuli can degrade it.

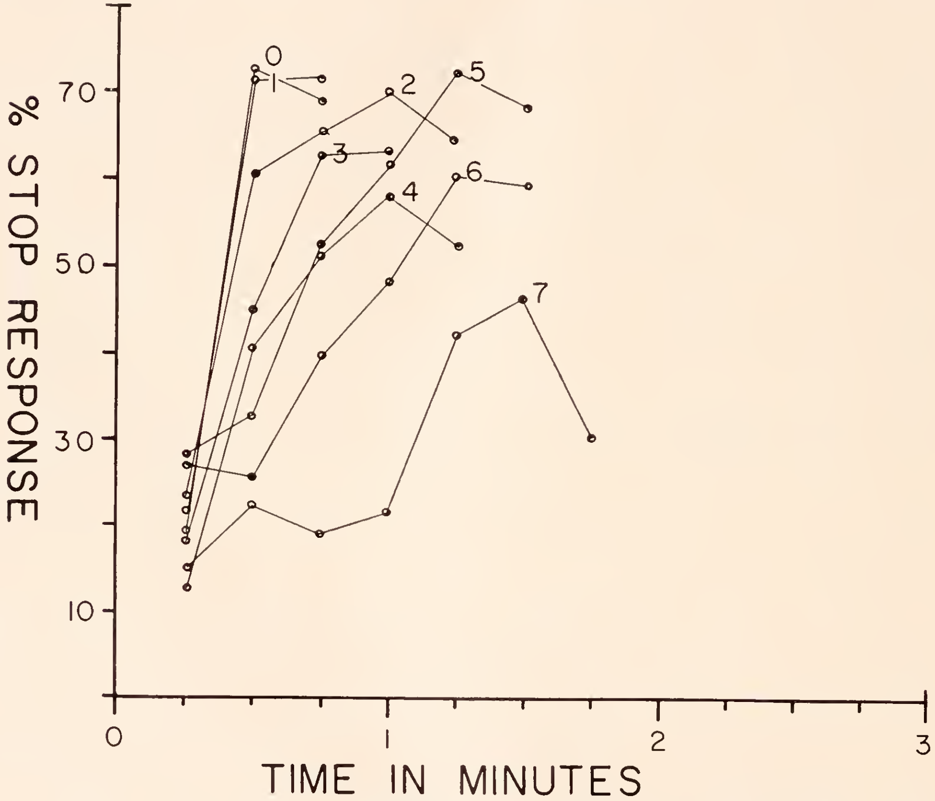

Figure 9. The relation of stimulus intensity to stop-response magnitude when light-adapted cells are placed in red light (600–700 mμ at 300 ft.-c.). The points represent the return to maximum response shown by 100 cells, when these cells are stimulated at different intensities. The numbers represent the different stimulus intensities as given in Figure 5 with two additions: 6 = 77 ft.-c., 7 = 66 ft.-c.

Discussion

On the basis of what is known about photoreception in animals, what can be said concerning the findings presented here? In the animal system, a pigment, rhodopsin, is changed from a photoactive state to a photo-inactive state through a rapid chemical isomerization. The process by which the active state is restored requires several enzymatic steps, and is quite slow (± 30 minutes in man) as demonstrated by the dark-adaptation phenomenon. Most important to this discussion, however, the return to sensitivity of the receptor system requires no photochemical steps. How does this system compare with that of *Gyrodinium*? We assume that a 470 mμ light-absorbing photoreceptor pigment shifts from an active to inactive state when subjected to this wavelength. The shift from active to inactive state triggers processes which ultimately result in a motor response. The entire process, from reception to behavioral act, requires 400 to 600 milliseconds, hence is quite fast. In this respect it resembles the animal system. The difference lies in the regeneration mechanism of photosensitivity. It appears that the regeneration time is short, possibly involving few, if any, enzymatic steps. The key to this short regeneration time lies in the obvious presence of a photochemical step, this being demonstrated by the red light effect. We feel that the red light is a necessary precursor for photo-response regeneration. This assumption is based on four findings. First, it was noticed that *Gyrodinium* would grow, but gave a poor phototactic response, when raised under cold white fluorescent light alone. This light is lacking in the longer wavelengths, emitting predominantly in the blue region of the spectrum. When incandescent light was added, the response increased greatly. Second, we again point out the striking difference attained in dark-adaptation by adding red light during the recovery phase. The third point concerns our ability to dark-adapt the cells in the *absence* of red light. It must be remembered that the intense white light, used to bleach the photoreceptor pigment, must affect both the red- and blue-light-absorbing pigments. If, however, the cells are bleached in blue light alone, the return to sensitivity is not observed when the cells are placed in darkness. Fourth, it is quite clear that spontaneous, rapid, loss of photoresponse sensitivity is observed if the cells are placed in darkness, and that the photoresponse can be maintained or re-established if red light is present.

In order to explain why dark-adaptation was observed in the absence of concurrent exposure to red light, two assumptions concerning the interaction of the red- and blue-light-absorbing systems during bleaching in white light must be made:

1. The blue-absorbing pigment must be inactivated at a rate which exceeds that rate at which it can be reactivated.
2. The red-absorbing system must produce some entity which is stable and produced in excess, if subsequent dark-adaptation in the absence of red light is to occur.

For these two opposing processes to occur, there must be some rate-limiting step present within the inactivation-activation phase. The confirmation of these assumptions lies with biochemical examination of pigment interaction in the cell. Before this is possible, the identity of the pigment or pigments must be determined.

The question remains as to just what the red-blue system represents in terms of the overall photoreceptor mechanism. One idea concerns a two-pigment system, with one pigment supplying energy or some entity necessary for the proper action of the other. It is possible that photosynthesis is involved with photoreception in this manner. Diehn and Tollin (1966) have suggested that photosynthesis is a necessary precursor for phototactic activity in *Euglena*. Their data do not indicate how direct this relationship may be with respect to the photoreceptor. A second possibility exists in a one-pigment system in which response is governed by a shift from a blue- to a red-absorbing state. With the introduction of red light, the pigment returns to the blue, response-active, state. Similarly acting pigments, the phytochromes, do occur in higher plants, and are responsible for the control of flowering and other processes. It is interesting to note that these pigments generally demonstrate peaks of absorption in the blue, red, and far-red regions of the spectrum.

An important point concerns the relationship of this red-blue system to the distribution of a natural population of cells. It has been observed by Hasle (1950) that dinoflagellates undergo a vertical migration, approaching the surface during the day and descending at night. We have observed *Gyrodinium* in extreme numbers at the surface of the water at mid-day. Observations at night indicate that this dense population descends or disperses, for the organisms cannot be found at the surface in the same concentrations. As our data indicate, the photoreceptor machinery loses sensitivity shortly after the onset of darkness. With the loss of sensitivity, movement of the cells becomes undirected relative to light, and they descend under the influence of gravity. As our data further indicate, the receptor sensitivity is recovered if red light is introduced. It is interesting to note that the predominant radiation at dawn exists in the red region of the spectrum. Full photoreceptor sensitivity can be regained, therefore, shortly after dawn, allowing subsequent migration toward the surface to occur. The red system may act as a "cocking" device for the phototactic response, while the response itself is "triggered" by blue light. One must keep in mind that the effects of red light on photoreceptor sensitivity studied here represent only short-term changes in the sensitivity level. Other long-term phenomena such as endogenous rhythmic control of light sensitivity may be present.

It would appear that the vertical distribution of these cells, and possibly of other phytoplankters, is a function of the level of photoreceptor sensitivity, which may change rapidly under varying wavelengths and intensities of illumination.

SUMMARY

1. The effect of blue light on the behavior of *Gyrodinium dorsum* is described. Action spectra for the orientation and "stop" responses are presented.

2. Dark-adaptation curves for the stop-response in darkness and red light are presented.

3. The maintenance effect of red light on these responses is described and a possible two-pigment machinery proposed.

4. The relationship of these phenomena to the vertical distribution of the phytoplankter in nature is discussed.

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HISTOCHEMICAL AND BACTERIOLOGICAL STUDIES ON DIGESTION IN NINE SPECIES OF LEECHES (ANNELIDA: HIRUDINEA)

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Previous studies on digestion in the leeches (summarized by Herter, 1936; Harant and Grassé, 1959; Mann, 1962) have been restricted to a few genera, notably *Hirudo* and *Haemopis.* Such accounts as are available, however, indicate that the full complement of digestive enzymes normally present in the alimentary system is, in the leeches, much reduced. In *Hirudo,* for example, the careful experiments of Graetz and Autrum (1935) failed to demonstrate proteases in gut wall extract, and this supported earlier observations by Diwany (1925) that starved *Hirudo* could not digest milk, egg proteins or peptones injected aseptically into the gut. With *Haemopis* Autrum and Graetz (1934) and Graetz and Autrum (1935) showed that enzymes for initiating proteolysis (the endopeptidases of modern terminology) are absent, but that a number of those concerned in subsequent stages (exopeptidases) are to be found in extracts of the gut wall.

The alimentary system in leeches shows well developed regional differentiation in that a pharynx, oesophagus, crop, intestine and rectum are generally present, but, in contrast, there is little or no differentiation at the cellular level into glandular and absorptive components. This apparent lack of gland cells, apart from salivary glands, lends support to the biochemical evidence that digestive enzymes are reduced in number, although such absence of intercellular differentiation does not *ipso facto* eliminate the possibility of secretory activity in the gastrodermis.

The failure to demonstrate a full range of proteolytic enzymes led Graetz and Autrum (1935) to suggest that bacteria may be concerned in digestive processes, and this possibility was subsequently examined by Büsing (1951) and Büsing, Döll and Freytag (1953). A single species of bacterium, named by Büsing *"Pseudomonas hirudinis,"* was found consistently in the gut lumen of *Hirudo* and *in vitro* studies showed that the microorganism is capable of slowly digesting the blood which is the normal food of this leech. Inclusion of antibiotics in the food *in vivo* inhibited digestion. *"P. hirudinis"* was subsequently shown to be a species of *Aeromonas,* probably *A. liquefaciens* (Bullock, 1961).

Close association of bacteria with the alimentary system has been reported for a number of leeches in addition to *Hirudo* and they occur either in special oesophageal diverticula or intracellularly within the gastrodermis (Reichenow, 1922; Jaschke, 1933; Hotz, 1938). The organisms have not been shown to have a specific role in digestion, but their fairly widespread occurrence in the Hirudinea suggests that bacterial involvement in digestive processes may be a feature of this class of annelids.

Accordingly, a study has been made of digestive processes in nine species of leeches representative of the four major families and of varying predatory or parasitic habits. Histochemical and substrate-film methods have been used to ascertain whether there is, in fact, a reduction in the complement of digestive enzymes, and to localize and identify any enzymes still produced. Standard bacteriological methods have been used in parallel studies to detect any microorganisms present in the gut lumen, to characterize them as far as possible, and to assess their possible role in digestion from their behavior *in vitro.*

Materials and Methods

The following species of leeches, listed systematically and with an indication of their basic mode of life, have been examined:

Family Hirudidae

Hirudo medicinalis L. Fresh-water sanguivorous parasite, sucking blood principally from mammals but also from reptiles or amphibia.

Family Erpobdellidae

Erpobdella octoculata (L.). Fresh-water predator.

Family Glossiphoniidae

Glossiphonia complanata (L.). Fresh-water predator.
Helobdella stagnalis (L.). Fresh-water predator.
Theromyzon tessulatum (O. F. Müller). Fresh-water parasite on water fowl, sanguivorous.
Hemiclepsis marginata (O. F. Müller). Fresh-water parasite on fish and Amphibia, sanguivorous.

Family Piscicolidae

Piscicola geometra (L.). Fresh-water parasite on fish, sanguivorous.
Pontobdella muricata (L.). Marine parasite on fish, sanguivorous.
Platybdella anarrhicae (Diesing). Marine parasite on fish, sanguivorous.

Starved individuals and others fed at varying intervals before examination were relaxed in 10% magnesium chloride and then subjected to histological, histochemical or bacteriological studies.

Histological methods

Specimens fixed in Bouin, Susa or 10% neutral formalin were embedded in polyester wax (Steedman, 1957) and sections cut at 4 μ stained by haematoxylin and eosin, Mallory, periodic acid-Schiff, Feulgen, and the benzidine method for haemoglobin (Pickworth, 1934).

Histochemical methods

Enzyme activity was studied using frozen or 45° C. paraffin wax sections prepared after fixation at 4° C. in 10% formalin buffered to pH 7.0.

1. Endopeptidases. A positive reaction to the Hess and Pearse (1958) method for cathepsin C-like esterases has been used by Jennings (1962a; 1962b) and Rosenbaum and Ditzion (1963) as an indication of the presence of endopeptidases since it coincided with, and explained, observed progressive proteolysis in the gut of various invertebrates. In the absence of more specific techniques for endopeptidases this was adopted in the present study, using *o*-acetyl-5-bromoindoxyl acetate as substrate (Holt and Withers, 1952) and with preincubation of sections in 10^{-5} *M* E600 (diethyl-*p*-nitrophenyl phosphate) at 37° C. for 1 hour to inactivate B-type esterases, including lipases, which would otherwise give false positive reactions.

2. Exopeptidases. Aminopeptidase activity was visualized using L-leucyl-β-naphthylamide hydrochloride as substrate and Garnet GBC as simultaneous coupler (Burstone and Folk, 1956). When frozen sections were used they were first defatted in acetone to avoid diffusion of the fat-soluble reaction product.

3. Lipases. Possible lipolytic activity was investigated by the Tween 80 method (Gomori, 1952) and by the Holt and Withers (1952) method for esterases.

4. Carbohydrases. The ferric hydroxyquinoline method for β-glucuronidase as modified by Fishman, Goldman and Green (1964) was used to detect carbohydrases.

5. Alkaline phosphatase. Metal-salt and azo-dye methods (Gomori, 1952) were used to detect alkaline phosphatase, using sodium β-glycerophosphate and sodium α-naphthyl phosphate, respectively, as substrates and Fast red TR as coupler.

6. Acid phosphatase. This enzyme, often associated with intracellular digestion and lysosomal activities, was visualized in sections by the azo-dye method (Burstone, 1958) using naphthol AS–BI or AS–TR phosphates as substrates and Red violet LB as coupler.

Controls for histochemical methods included heat-inactivated sections, media lacking specific substrates, and the simultaneous processing of appropriate mammalian tissues.

Substrate-film methods

Thick frozen sections or bisected whole leeches, prepared after brief fixation in cold 10% neutral formalin, were tested for proteases by the silver halide-gelatine film method (Adams and Tuqan, 1961), for amylases by the starch-film method (Tremblay, 1963), and for lipases by laying upon tributyrin agar plates (Willis, 1960). Incubation at 22° C. was for 1 hour only, and heat-denatured material and drops of Seitz-filtered solutions of commercially available enzymes served as controls.

Bacteriological methods

1. Cultivation of the gut flora. The largest available individuals of each species were relaxed, pinned out and a hot soldering iron or scalpel used to sterilize the body surface over the gut region to be sampled. Ligatures were used to isolate regions of the gut, and on occasion the gut was exposed by dissection and its outer surface sterilized. Samples of gut contents were removed by forcing a fine Pasteur

pipette through the sterilized surface, and spread on nutrient agar. The agar plates were incubated at 25° C. for 24 hours together with controls inoculated from the sterilized surfaces, and only cultures whose corresponding controls showed no growth were retained for further study.

2. Hydrolytic capacities of the gut flora. The total hydrolytic effect of extracellular enzymes produced by microorganisms isolated from the gut was assessed qualitatively by culturing them, still as mass cultures not separated into pure strains, on appropriate media. Proteolytic activity was detected by blood agar, chocolate agar, skim milk agar, gelatine discs impregnated with charcoal, and Loeffler's serum. Lipases were detected by agar containing egg yolk, tributyrin or Tween 80 (Sierra, 1957) and amylases by starch agar. Blood agar served also for detection of haemolysins and egg yolk agar for lecithinases. Seitz-filtered solutions of commercial enzyme preparations acted as controls.

3. Characterization of components of the gut flora. Pure cultures were obtained by repeated subculturing, as necessary, from the original stocks and tested to gain some idea of the main types present. The tests used included growth on nutrient agar, blood agar and Loeffler's serum; nitrate reduction and citrate utilization; production of hydrogen sulfide, urease, catalase, oxidase or indol; the Voges Proskauer test; gelatine liquefaction; aesculin breakdown; growth in media containing glucose, lactose, sucrose, or starch; and sensitivity to penicillin and streptomycin. Details of these tests may be found in any standard bacteriology text.

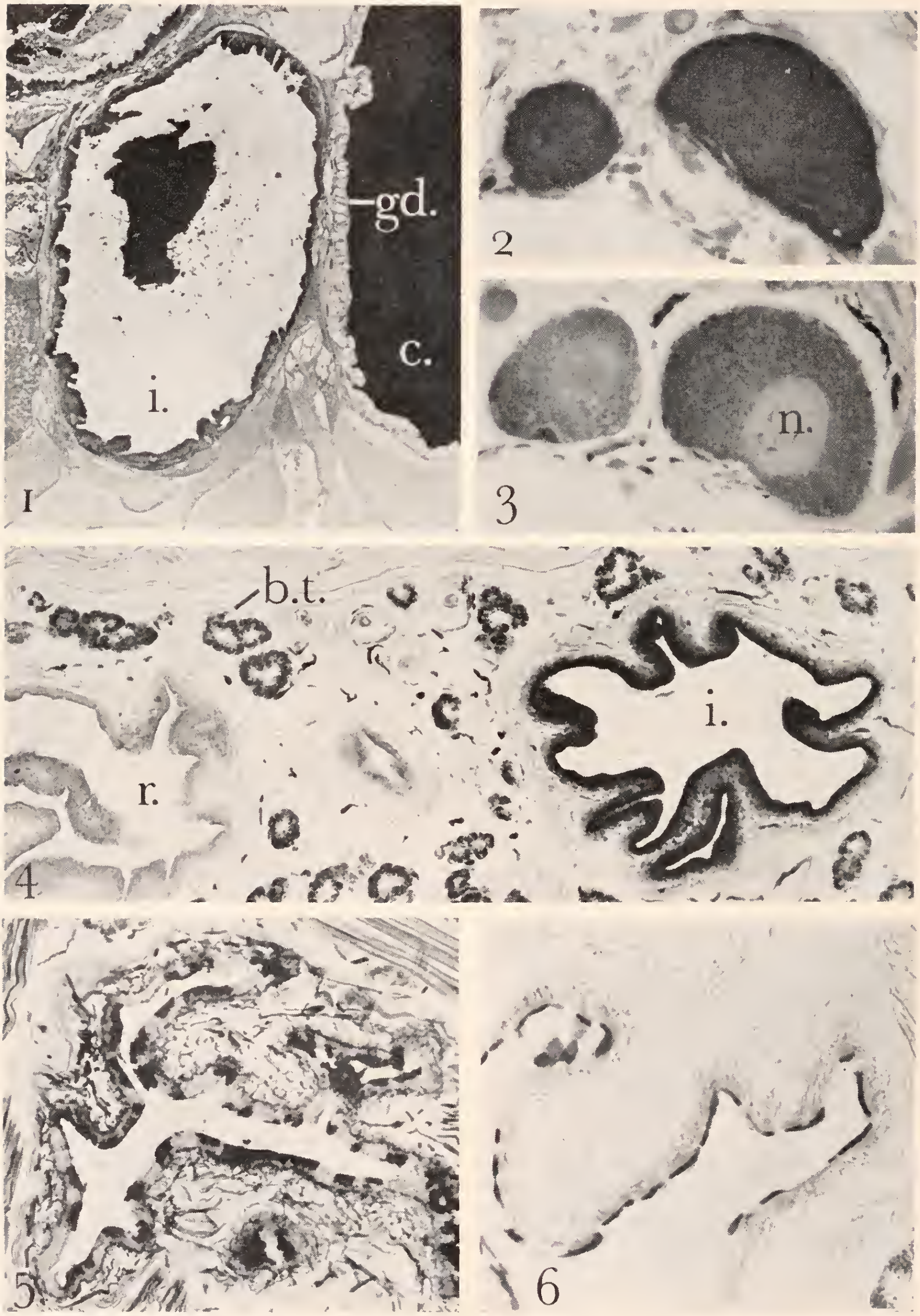
As controls *Pseudomonas fluorescens* was subjected to each series of tests, and other named strains were used in some individual tests. The tests selected included the majority of those used by Büsing (1951) to characterize *"P. hirudinis."*

Observations

General observations on feeding, gut structure and digestion

The six parasitic species feed on blood drawn from the respective hosts but the three predators feed on a variety of small invertebrates such as oligochaetes, other leeches, molluscs or insect larvae. *E. octoculata* swallows its prey intact but *H. stagnalis* and *G. complanata* use the proboscis to suck out fluids and soft tissues. The food is passed to the crop where it remains for periods varying from 1 to 2 days (*H. stagnalis*) to 6 months or more (*T. tessulatum*). Histological examination showed that there is no differentiation of the gastrodermis, in any of the species examined, into glandular and other components such as occurs in other annelids. The gastrodermis is relatively uniform in structure and there is no significant difference between that of the crop and intestine. A striated border, which stains strongly with the periodic acid-Schiff technique, is consistently present in both regions.

The course of digestion was traced in both parasitic and predatory species by following the fate of blood meals, the predators being induced to feed on clotted frog blood. Digestion in the gut lumen resulted in visible breakdown of erythrocytes, lysis of their nuclear membranes and release of nuclear constituents demonstrable in early stages by the Feulgen reaction but subsequently disappearing, and the degradation of haemoglobin into brown insoluble granules of haematin. In two species, *P. geometra* and *H. stagnalis,* there is extensive digestion in the crop


gd.
c.
i.
1
2
3
n.
b.t.
r.
i.
4
5
6

Figures 1–6.

but in all the others the significant amount of digestion occurs in the intestine, food being passed into this region a little at a time from the crop. Digestion in the intestine is predominantly extracellular but in *E. octoculata, G. complanata, T. tessulatum* and *H. marginata* the appearance of haem compounds in the gastrodermis (Fig. 1) suggests that there is also some intracellular digestion following absorption of materials from the gut lumen.

Occurrence and distribution of enzymes in the gut

1. Endopeptidases

Endopeptidases, as tested for by the Adams and Tuqan substrate-film method for proteases in general and the more specific histochemical method for E600-resistant esterases, could not be located in any part of the leech gut apart from in *P. geometra.* In this leech alone a large amount of esterase activity, some of it E600-resistant, occurs in the salivary glands (Figs. 2 and 3) and in the crop contents of recently fed individuals. None is produced by the crop gastrodermis, however, and that found amongst crop contents presumably originates in the salivary glands since leeches commonly inject saliva into host tissues during ingestion of the food. As noted earlier, digestion in *P. geometra* occurs largely in the crop and is much more rapid than in other sanguivorous species, erythrocytes which are prominent in the crop soon after feeding being completely digested within 10 days. It seems likely that salivary esterase is at least partly responsible for this breakdown.

In *P. anarrhicae* esterase activity was absent from the salivary glands but could be found on occasion in crop contents. Unlike the situation in *P. geometra*, however, activity was abolished by pre-treatment with E600. *P. anarrhicae* lives in the gill chamber of marine catfish and feeds principally on blood drawn from the gill capillaries, but some gill tissue and mucus are also ingested. Control sec-

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FIGURE 1. *Theromyzon tessulatum.* Transverse section showing part of a diverticulum of the crop (c.) which projects posteriorly alongside the intestine (i.). The crop is filled with haemolyzed blood but its gastrodermis (gd.) shows no trace of absorbed haem compounds. In contrast there is little blood in the intestine and the intestinal gastrodermis is loaded with haem compounds absorbed from the lumen. Pickworth benzidine method for haemoglobin and haem compounds. Scale: 1 cm. = 100 μ.

FIGURE 2. *Piscicola geometra.* Transverse section through two salivary glands, both of which show an intense positive reaction for esterase. Hess and Pearse method without preincubation in E600. Scale: 1 cm. = 50 μ.

FIGURE 3. *Piscicola geometra.* Transverse section through the same salivary glands as in Figure 2, but showing some inhibition of esterase activity caused by preincubation of the section in E600. n., nucleus of gland cell. Hess and Pearse method with preincubation for 1 hour at 37° C. in 10^{-5} *M* E600. Scale: 1 cm. = 50 μ.

FIGURE 4. *Erpobdella octoculata.* Longitudinal section passing through the rectum (r.) and intestine (i.). The intestinal gastrodermis shows a strong positive reaction for aminopeptidase. The dark color of the botryoidal tissue (b.t.) is due to its endogenous pigmentation and does not represent a positive reaction. Burstone and Folk method. Scale: 1 cm. = 100 μ.

FIGURE 5. *Erpobdella octoculata.* Transverse section of the intestine showing the distribution of alkaline phosphatase in the gastrodermis. Gomori azo-dye method. Scale: 1 cm. = 50 μ.

FIGURE 6. *Erpobdella octoculata.* Longitudinal section showing the distribution of acid phosphatase in the striated border of the intestinal gastrodermis. Burstone azo-dye method. Scale: 1 cm. = 50 μ.

tions showed that gill tissue itself contains some non-specific and E600-sensitive esterase and this presumably persists for a time after ingestion by the leech. An identical occurrence of non-specific esterase of host origin has been recorded in the gut of the gill-dwelling monogenetic trematodes *Diclidophora merlangi* and *Octodactylus palmata* (Halton and Jennings, 1965).

In the majority of leeches prolonged incubation for 24 hours or more in the bromoindoxyl acetate medium produced scattered and very weak positive reactions in the intestinal gastrodermis. This was abolished by pre-treatment in E600, showing that the enzyme responsible is probably a B-type esterase and not a digestive endopeptidase.

2. *Exopeptidases*

In marked contrast to the negative results obtained for endopeptidases an extremely strong positive reaction to the Burstone and Folk method for aminopeptidase was given by the intestinal gastrodermis of all species except *H. medicinalis* and *P. muricata,* where somewhat weaker reactions occur. In the other seven species the intestinal gastrodermis shows an intense and continuous positive reaction (Fig. 4), and the most striking feature of this is the fact that it is always present irrespective either of the time elapsed since the previous meal or of the presence or absence of food in the lumen. It was found in every individual examined and was demonstrated with equal ease in both frozen and wax sections.

Material lying in the intestinal lumen on occasion showed a strong positive reaction, comparable to that of the gastrodermis. This extracellular activity clearly originated from the intestinal gastrodermis since neither the crop gastrodermis nor crop contents showed any trace at any time.

It is possible that the weak E600-sensitive esterase reaction given by the intestinal gastrodermis after prolonged incubation is caused by an aminopeptidase, as Smith and Hill (1960) state that pure leucine aminopeptidase shows slight esterase activity when tested biochemically against a number of substrates. On the other hand, Holt (1963) claims that this enzyme is resistant to E600 and if this is correct then the weak esterase activity of the leech intestine must be due to other, unidentified, causes.

3. *Lipases*

It was thought that lipases may have been partly responsible for the weak esterase activity found in the intestinal gastrodermis, since the reaction proved sensitive to E600, but Gomori's Tween 80 method failed to reveal any lipolytic activity anywhere in the gut, in any species. With the substrate-film method, frozen sections or bisected individuals of *G. complanata* and *P. geometra* caused slight clearing of tributyrin agar but the activity could not be localized to any one region of the gut.

4. *Carbohydrases*

No reaction was obtained to either substrate-film or histochemical methods for carbohydrases in any part of the gut, although the body musculature in most species gave positive results to tests for β-glucuronidase.

5. *Alkaline phosphatase*

Alkaline phosphatase, as demonstrated by the metal-salt and azo-dye methods, was found in the crop and intestinal gastrodermis in all species. Its distribution is somewhat patchy (Fig. 5) in that groups of cells show a reaction but these are interspersed with others showing little or no activity. Generally, the entire cytoplasm is involved in the cells showing activity, and there is no significant difference between cells of the crop or intestine.

The occurrence and distribution of alkaline phosphatase within the gastrodermis is quite independent of the nutritive state of the animal, precisely as in the case of aminopeptidase.

Slight reactions were obtained in the salivary glands of *H. medicinalis, T. tessulatum* and *P. geometra* and in the last two species the enzyme occurs also in the integument over the entire body except for the adhesive surfaces of the suckers.

6. *Acid phosphatase*

The distribution of acid phosphatase, as traced by the Burstone azo-dye method, followed closely that of alkaline phosphatase except that it could not be demonstrated in the crop of *H. marginata.* The enzyme occurs generally in the striated border, but occasionally extends well into the distal half of the cell. The distribution throughout the gastrodermis is discontinuous (Fig. 6), as in the case of alkaline phosphatase, but, again, it is consistently present irrespective of the presence or absence of food.

In *P. geometra* the salivary glands, which produce the salivary esterase, show at all times a strong reaction for acid phosphatase, and in *T. tessulatum* occasional cells in the salivary glands of young individuals show a similar response.

Bacteriological results

1. *Hydrolytic capacities of the gut flora*

Eight leech species were investigated in this part of the study, *H. stagnalis* being omitted since insufficient specimens were available. Samples from crop and intestine were obtained separately whenever possible but in some instances it was impossible to state categorically that samples came from one region alone, due to the size of the animal or movement of gut contents prior to ligaturing and sterilization. Cultures obtained from samples withdrawn under these circumstances are designated in the summaries of results as from the "gut," as opposed to others known to originate specifically in the crop or intestine.

The number of attempts to establish mass cultures from the different regions of the gut, and the number of successes, are summarized in Table I. Various factors probably account for the fairly high proportion of failures but a significant one, no doubt, was sterilization of gut contents during the essential sterilization of tissues prior to removal of samples.

The results of testing the mass cultures by each of five methods for proteases, three for lipases and one for amylases are summarized in Tables II and III. Table IV summarizes results of tests for haemolysins which produced β-haemolysis type color changes on blood agar, and tests for lecithinases on egg yolk agar.

TABLE I

Summary of attempts to establish mass cultures of bacteria from the leech gut
(a., no. of attempts; s., no. of successes)

	"gut"		crop		intestine		total	
	a.	s.	a.	s.	a.	s.	a.	s.
H. medicinalis	12	6	6	6	2	2	20	14
E. octoculata	16	12	9	3	11	7	36	22
G. complanata	9	8	7	7	6	5	22	20
T. tessulatum	16	8	27	6	2	2	45	16
H. marginata	2	2	5	2	1	1	8	5
P. geometra	12	4	16	7	1	0	29	11
P. muricata	—	—	2	2	—	—	2	2
P. anarrhicae	18	4	—	—	—	—	18	4

TABLE II

Summary of tests for proteases on mass cultures of gut bacteria

When only a proportion of the cultures established were tested the number used is shown after the number of positive results, otherwise the total number established were tested.
b.a., blood agar; c.a., chocolate agar; m.a., milk agar; c.g. charcoal gelatine; L.s. Loeffler's serum.

	Cultures established	Nos. effecting hydrolysis on					Overall %
		b.a.	c.a.	m.a.	c.g.	L.s.	
H. medicinalis							
"gut"	6	6	6	6	6	6	100%
crop	6	6	6	6	6	6	(70:70)
intestine	2	2	2	2	2	2	
E. octoculata							
"gut"	12	10:10	2:2	8:8	10:10	8:8	100%
crop	3	3	3	3	3	3	(84:84)
intestine	7	7	5:5	6:6	6:6	7	
G. complanata							
"gut"	8	6:6	6	7	5:7	8	87%
crop	7	7	4:6	5	5:6	7	(83:95)
intestine	5	5	5	5	4	4	
T. tessulatum							
"gut"	8	4	2:4	5	7	4:4	57%
crop	6	4	1	2:2	2	5	(38:66)
intestine	2	2	0	0	0		
H. marginata							
"gut"	2	2	1	2	2	1:1	95%
crop	2	2	2	2	2	2	(23:24)
intestine	1	1	1	1	1	1	
P. geometra							
"gut"	4	4	4	4	4	4	84%
crop	7	7	3	7	3	3:3	(43:51)
P. muricota							
crop	2	2	1	2	2	—	87%
							(7:8)
P. anarrhicae							31%
"gut"	4	2	1	0	0:2	2:2	(5:16)

If the organisms tested play a dominant part in the digestive processes of their hosts, then a success rate approaching 100% would be expected, *i.e.*, it is reasonable to expect each replicate mass culture such as the six established from crop contents of *H. medicinalis* to show a hydrolytic effect on each substrate. Controls of Seitz-filtered bacteria-free solutions of commercial pepsin, pancreatin and lipase did, in fact, give this expected 100% success rate.

TABLE III

Summary of tests for lipases and amylases on mass cultures of gut bacteria
e.y.a., egg yolk agar; t.a., tributyrin agar; Tw. a., Tween 80 agar; st.a., starch agar.

	Cultures established	Nos. effecting hydrolysis on				Overall % for	
		e.y.a.	t.a.	Tw.a.	st.a.	lipases	amylases
H. medicinalis							
"gut"	6	6	6	6	6		
crop	6	6	6	6	6	100%	100%
intestine	2	2	2	2	2	(42:42)	(14:14)
E. octoculata							
"gut"	12	12	12	12	12		
crop	3	3	3	2	3	97%	100%
intestine	7	6	7	7	7	(64:66)	(22:22)
G. complanata							
"gut"	8	5:6	4:5	5:7	8		
crop	7	4	6	4:6	7	79%	100%
intestine	5	4	5	5	5	(42:53)	(20:20)
T. tessulatum							
"gut"	8	1	3	1	0		
crop	6	2:5	6	1:5	1	39%	6%
intestine	2	0	2	2	0	(18:46)	(1:16)
H. marginata							
"gut"	2	2	2	2	0		
crop	2	1	2	2	1	86%	40%
intestine	1	1	1	0	1	(13:15)	(2:5)
P. geometra							
"gut"	4	2:2	2:3	2:2	4	96%	36%
crop	7	6:6	6:6	7	0	(25:26)	(4:11)
P. muricata							
crop	2	1	2	2	1	83% (5:6)	50% (1:2)
P. anarrhicae							
"gut"	4	2	3	0	0	41% (5:12)	0 (0:4)

The results show that in two species, *H. medicinalis* and *E. octoculata*, there is substantial evidence for the presence of a gut flora capable of taking a leading part in the leech's digestive physiology. Cultures from *H. medicinalis* gave scores of the expected 100% in tests for proteases, lipases and amylases, and those from

E. octoculata were the same apart from a score of 97% (64 positives out of 66 tests) for lipases. The other species, however, provided cultures showing varying degrees of discrepancy. Material from *G. complanata,* for example, gave relatively high scores (87% for proteases, 79% for lipases and 100% for amylases), but with *T. tessulatum* the score was only 57%, 30% and 6%, respectively, and with *P. anarrhicae* it was 31%, 41% and nil.

TABLE IV

Summary of tests for haemolysins and lecithinases on mass cultures of gut bacteria

	Cultures established	Numbers positive for		Overall % for	
		haemolysins	lecithinases	haemolysins	lecithinases
H. medicinalis					
"gut"	6	6	1		
crop	6	6	6	100%	64%
intestine	2	2	2	(14:14)	(9:14)
E. octoculata					
"gut"	12	10:10	9		
crop	3	3	0	100%	54%
intestine	7	5:5	3	(18:18)	(12:22)
G. complanata					
"gut"	8	5:5	0:6		
crop	7	3:5	2:5	80%	25%
intestine	5	4	2	(12:15)	(4:16)
T. tessulatum					
"gut"	8	3	0		
crop	6	1:3	1:5	30%	6%
intestine	2	0	0	(4:13)	(1:5)
H. marginata					
"gut"	2	0	0		
crop	2	0	2	20%	60%
intestine	1	1	1	(1:5)	(3:5)
P. geometra					
"gut"	4	4	1	36%	54%
crop	7	0	5	(4:11)	(6:11)
P. muricata					
crop	2	0	0	0 (0:2)	0 (0:2)
P. anarrhicae					
"gut"	4	2	1	50% (2:4)	25% (1:4)

The complete conformity with the expected 100% in the *H. medicinalis* cultures and in the majority of those from *E. octoculata* suggests that the nonconformity of figures from the other six species is significant. Thus it would appear that while populations of bacteria are present which possess considerable hydrolytic abilities it seems unlikely that the microorganisms participate in digestive activities to the same extent as in *H. medicinalis* or *E. octoculata.* Nevertheless, their hydrolytic abilities are such that the possibility of their participation to some lesser extent cannot be denied.

The conclusion that the gut bacteria cannot be the sole agent in the digestive processes of most leeches is supported by the fact that their hydrolytic abilities vary considerably both between and within host species. In *T. tessulatum,* for example, cultures established from different individuals do not always affect blood agar, chocolate agar, milk agar or charcoal gelatine; thus there are no grounds for asserting that in *T. tessulatum* the gut flora at all times possesses components which together can attack a wide range of proteinaceous substrates. Similarly, in *P. geometra* only blood agar, milk agar and Loeffler's serum were consistently attacked by cultures from all animals tested, and the frequency with which other substrates were attacked varied considerably between individuals.

The activities designated in Table IV as "haemolysins" and "lecithinases" have not been included in the above observations since the enzymes responsible represent specialized classes of proteases and lipases, respectively. Both types could conceivably be of importance in the digestive physiology of sanguivorous leeches, with lecithinases concerned in breakdown of erythrocyte walls and haemolysins being perhaps especially important in degradation of haemoglobin. Thus if the gut flora participated extensively in digestive processes it could be expected that all cultures would show these activities, at least in sanguivores. *H. medicinalis,* however, was the only sanguivore yielding cultures showing 100% positives for haemolysins. The same value was obtained for lecithinases in cultures known to have originated from either the crop or intestine, but of those from the "gut" only 1 out of 6 were positive. Inclusion of these "gut" results reduces the total incidence of positives for these enzymes in *H. medicinalis* to 64%.

E. octoculata, a predator and non-sanguivore, resembles *H. medicinalis* in respect of the high haemolysin and lecithinase activities of its gut flora, but in the other leeches these activities are more limited and even in sanguivores the score was consistently low. In *T. tessulatum* and *P. geometra,* for example, the score for haemolysins was only 30% and 36%, respectively.

2. *Characterization of components from the gut flora*

A full bacteriological investigation of the flora of the leech gut was beyond the scope of the present study, but a number of standard tests were applied to pure cultures of Gram-negative rods isolated from "gut" samples of *H. medicinalis, E. octoculata, T. tessulatum, H. marginata* and *P. geometra.* A synopsis of the tests and results obtained is given in Table V. Interpretation of the results is based on data given by Breed, Murray and Smith (1957) ; Hugh and Leifson (1953) ; Klinge (1960) ; Park (1962) ; Rhodes (1959) ; and Shewan, Hobbs and Hodgkiss (1960).

A number of the pure strains broke down Hugh and Leifson's glucose medium oxidatively, *i.e.* under strictly aerobic conditions. This is a characteristic of most species of *Pseudomonas* and strains ER1 and ER2 (*E. octoculata*), HM1 and HM2 (*H. marginata*), and PI1 and PI2 (*P. geometra*) are identified as such, their other properties as summarized agreeing with this. Strains TH1 and TH2 (*T. tessulatum*) also attacked Hugh and Leifson's medium oxidatively, but are not identified as pseudomonads as they failed to utilize citrate. They are tentatively identified as species of *Xanthomonas* since although they lack the characteristic pigmentation their properties agree best with *Xanthomonas* data.

The majority of the remaining strains broke down Hugh and Leifson's medium by fermentation, with production of gas, indicating *Aeromonas* sp. They included HR1 and HR2 from *H. medicinalis* and this identification agrees with data given by Bullock (1961) for the identification of *"Pseudomonas hirudinis"* (Büsing, 1951) as an *Aeromonas,* probably *A. liquefaciens.* Strains ER3, ER4 and ER5 (*E. octoculata*) were the others effecting fermentation and identified on the basis of this and other properties as *Aeromonas* sp.

TABLE V

Synopsis of tests on pure cultures from the leech gut

HR *H. medicinalis;* ER *E. octoculata;* TH *T. tessulatum;* HM *H. marginata;* PI *P. geometra.*

	HR		ER						TH		HM		PI	
	1	2	1	2	3	4	5	6	1	2	1	2	1	2
Gram stain	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Mobility	+	+	+	+	+	+	+	−	+	+	+	+	−	−
Polar flagella	1	1	3	3	1	1	1	−	1	1	1	1	3	3
Reaction in media containing:														
glucose (H. & L.)	F	F	Ox.	Ox.	F	F	F	Ox.	Ox.	Ox.	Ox.	Ox.	Ox.	Ox.
lactose	AG	AG	−	−	−	−	−	−	A	A	−	−	−	−
sucrose	AG	AG	−	−	A	A	A	−	A	A	−	−	−	−
Starch hydrolysis	+	+	−	−	+	+	−	−	−	−	−	−	−	−
Aesculin breakdown	+	+	−	−	−	+	+	−	+	−	−	−	−	−
Citrate utilization	+	+	+	+	+	+	+	+	−	−	+	+	+	+
Nitrate reduction	+	+	−	+	+	+	+	+G	+	−	+	−	−	−
Formation of:														
indol	−	−	−	−	−	−	−	−	−	−	−	−	−	−
H_2S	+	+	−	−	+	+	+	−	+	+	−	−	−	−
urease	−	−	+	+	+	+	−	−	−	−	−	−	−	−
oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pigment	−	−	Gr.	Gr.	−	−	−	−	−	−	−	−	−	−
Gelatine liquifactn.	+	+	+	−	+	+	+	−	+	+	+	+	+	+
Voges Proskauer	+	+	−	−	+	−	+	−	−	−	−	−	−	−
β haemolysis	+	+	−	+	+	+	+	−	+	+	+	+	−	−
Fluorescence in U.V.	−	−	+	+	−	−	−	−	−	−	+	+	+	+
Sensitivity to:														
penicillin	−	−	−	−	−	−	−	−	−	−	−	−	−	−
streptomycin	+	+	+	+	+	+	+	−	−	+	+	+	+	+

−, negative; +, positive; F, fermentation with gas production; Ox., oxidative breakdown; A, acid production; G, gas production; Gr., green pigment produced.

The remaining strain from *E. octoculata,* ER6, was an encapsulated form producing abundant slime, and is identified as a species of *Klebsiella.*

The principal fact emerging from these tests, then, is that the predator *E. octoculata* harbors more species of microorganisms than the sanguivorous leeches examined, all of which yielded only one species. Further, the types obtained from the sanguivores varied with the particular host species, *H. medicinalis* yielding only *Aeromonas* sp., *H. marginata* and *P. geometra* giving species of *Pseudomonas*, and *T. tessulatum* species of *Xanthomonas*. In contrast, *E. octoculata* yielded species of both *Pseudomonas* and *Aeromonas*, as well as of *Klebsiella*.

All these types of bacteria are commonly found in the fresh-water habitat of the various leeches.

DISCUSSION

Previous reports that the gastrodermis in leeches lacks morphological differentiation into secretory and absorptive components have been substantiated in the present study, where histological and histochemical examinations have failed to reveal any glandular structures. Further, in contrast to most other animals, the leeches do not appear to produce endopeptidases in any part of the alimentary system, apart from in the isolated case of *P. geometra* where an E600-resistant esterase forms part of the salivary secretion. It is impossible, however, to state categorically that endopeptidases are absent, since Vanha-Pertula, Hopsu, Sonninen and Glenner (1965) have shown that the endopeptidase cathepsin C when freed from all impurities fails to hydrolyze significant amounts of the bromoindoxyl acetate which was used as a substrate in the search for these enzymes in the leech. It is unlikely, though, that the leech gastrodermis would produce a protease of this type alone, and the techniques used here have certainly revealed endopeptidases or endopeptidase-like enzymes in other invertebrate groups (Jennings, 1962a, 1962b; Rosenbaum and Ditzion, 1963).

In marked contrast to the apparent absence of endopeptidases a strong positive reaction to the Burstone and Folk method for aminopeptidase is universally obtained in the intestinal gastrodermis. This reaction is, in fact, indicative of the presence of several exopeptidases, including leucine aminopeptidase for which the method was originally believed to be specific, as it has been shown that the L-leucyl-β-naphthylamide substrate can be split by a range of amino-peptidases and by at least one carboxypeptidase (Sylvén and Bois, 1962, 1963; Sylvén and Bois-Svensson, 1964). Such occurrence of exopeptidases in the gastrodermis at all times and hence independently of the nutritive state of the leech is quite different from the situation in other invertebrates such as flatworms and nemerteans where an aminopeptidase reaction can be demonstrated only at specific times after feeding (Rosenbaum and Rolón, 1960; Jennings, 1962a, 1962b). In these animals the reaction is entirely intracellular but in the leeches it is also found extracellularly, and this may account for the fact that the enzymes responsible can always be demonstrated in the intestinal gastrodermis. There is strong evidence that the exopeptidases are concerned in intracellular digestion, as well as extracellular, since haem compounds were found in the gastrodermis, apparently undergoing digestion, in a number of cases.

The occurrence of acid and alkaline phosphatases, like that of the exopeptidases, is unrelated to the nutritive state of the leech. These enzymes are probably concerned with absorption of material from the gut lumen, as they occur consistently in the striated border of both crop and intestinal cells. In the latter the phosphatases are probably concerned in uptake of materials for intracellular digestion, but their role in the crop gastrodermis is less clear. In sanguivores water, salts and soluble metabolites such as glucose may be absorbed from the blood meal as it lies in the crop, and in *P. geometra* there is probably absorption of products of the digestion effected in the crop by salivary esterase. The crop is primarily a storage organ but in most animal groups possessing such a structure a few members have extended its use to include digestion effected by enzymes either swallowed as part of the saliva or regurgitated from the intestine (Jennings, 1965), and it is interesting to find that the leeches are no exceptions to this general rule.

The evidence that exopeptidases are the only endogenous proteases in the species examined agrees with similar findings for *Haemopis* by Autrum and Graetz (1934) and Graetz and Autrum (1935), and supports the idea that this may be characteristic of the Hirudinea as a whole. This, however, poses the problem of the mechanism of proteolysis which obviously cannot follow the same course as in other animals where endopeptidases and exopeptidases act in sequence. The hypothesis of bacterial involvement has been tested and in two species there is convincing evidence that microorganisms may be extensively involved in digestive processes, including proteolysis. In the other species the gut flora possesses more limited hydrolytic abilities and presumably, therefore, participates to a lesser extent in digestion. It is conceivable that the exopeptidases of the leech continue and complete proteolysis initiated by the gut flora, but it is perhaps more likely that the principal role of these enzymes is independent of the gut symbionts. Thus digestion of protein may well be effected by a series of exopeptidases, of various group and bond specificities, which between them progressively remove terminal amino residues from the protein chains. This type of activity has, in fact, been demonstrated *in vitro* by Hill and Smith (1958, 1959, 1960) who showed that pure leucine aminopeptidase can remove, stepwise, 109 of the 185 amino acid residues of papain and hydrolyze completely the polypeptide glucagon. Comparable activity in animal digestive processes would be slow and inefficient without initial intervention of endopeptidases to provide a greater number of terminal units for exopeptidase attack, but, in favor of this hypothesis, digestion in leeches *is* a slow and much extended process.

Support for this interpretation of the basis of leech digestive physiology can be obtained, paradoxically, from *H. medicinalis* where the gut flora seems to be capable of extensive participation in digestion. In this leech aminopeptidase activity in the gastrodermis is relatively weak, indicating perhaps that more reliance is placed on the gut flora than on endogenous enzymes. In *E. octoculata,* the other species in which bacteria could be extensively concerned in proteolysis, there is the more typical strong reaction for endogenous aminopeptidase so that in this species perhaps both types of mechanism operate. In *E. octoculata* a more varied gut flora is present, but this is probably related to the predaceous feeding habits which allow a wide selection of microorganisms to enter the gut with each meal and, no doubt, some of these become established in the new habitat.

Since no endogenous amylases or lipases have been found in the leech gut it is possible that the bacterial flora is concerned in digestion of these substances. The mass culture tests showed only restricted amylolytic and lipolytic activities, but carbohydrates and fats do not preponderate in the diets of either predatory or parasitic leeches. It seems obvious that bacteria are important in leech nutrition, in view of their fairly general occurrence (Reichenow, 1922; Jaschke, 1933; Hotz, 1938), and in addition to their suggested participation in digestion they may also contribute vitamins, probably of the B group, to the host economy since these will be scarce in the diet of sanguivorous leeches, if not in that of predators. A comparable situation exists in sanguivorous arthropods (summarized by Wigglesworth, 1965), where symbionts contribute significant quantities of vitamins to their hosts.

If this interpretation of the present evidence is correct, then it would appear that the leeches during their evolution have for some reason lost the capacity to produce endopeptidases, and probably also lipases and amylases. In compensation for lack of endopeptidases production of exopeptidases has been emphasized and these enzymes, aided by a symbiotic gut flora, have become responsible for the entire sequence of proteolysis. As a further adaptation the leeches have developed a tolerance of the consequent slowing down and extension of digestion, and adaption of sanguivorous habits with considerable lengths of time elapsing between meals has fitted in well with this type of digestive physiology.

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SUMMARY

1. Histological, histochemical and bacteriological methods have been used to study digestion and the nature and source of digestive enzymes in nine species of leech.

2. The gastrodermis is not differentiated morphologically into secretory and absorptive structures and there is little difference in the structure of the crop or intestine.

3. Endopeptidases, which initiate proteolysis in most animals, lipases and amylases do not appear to be produced by the leech digestive system.

4. The possibility that the gut flora is concerned in digestion in compensation for the lack of endogenous enzymes, has been investigated using mass cultures of the microorganisms normally present in crop and intestine. In two species the combined hydrolytic capacities of the gut flora are considered sufficient for it to play an important part in digestion of proteins, fats and carbohydrates; and in the other species there is evidence that it can participate to a lesser but still significant extent.

5. Exopeptidases, as typified by the presence of aminopeptidases, are produced in the intestinal gastrodermis and can be consistently demonstrated irrespective of the nutritive state of the animal. There is evidence that they act both intra- and extracellularly.

6. It is suggested that the exopeptidases play a part different from their normal one in animal digestive physiology, in that they act in the absence of endopeptidases by slowly degrading protein chains by progressive removal of terminal units. This proteolysis supplements any effected by the gut flora.

7. Acid and alkaline phosphatases are consistently present in the gastrodermis of the leech and are believed to be concerned with absorption of material from the gut lumen.

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DEVELOPMENTAL MODIFICATIONS IN ARBACIA PUNCTULATA BY VARIOUS METABOLIC SUBSTANCES[1]

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Normal development is a sequela of normal cellular differentiation. In order to understand the precise programming in development, be it molecular or morphologic, attention must be given to the properties of the individual developing cell and its organelles and its metabolic events, as well as the overall susceptibility of response of the whole organism during development.

Major steps in the understanding of echinoid developmental biology have been made by experimental approaches which have brought about aberrations in development (see review by Lallier, 1964a) and more recently by biochemical analyses of these events (see review by Monroy and Maggio, 1964).

The present report concentrates on the response of the embryo as a whole organism to a variety of agents. By comparison with the normal sequences in development, these changes may provide insight into the mode of action, especially where a differential response is elicited.

The first experiments were based on Heath's (1954) observation that persistent nucleoli resulted when 1 to 1.5 × 10^{-5} gm./ml. cobalt chloride was added to cultures of chick heart and frontal bone. These persistent nucleoli remained in association with chromosomes during metaphase but were torn in two or adhered to one chromosome at anaphase. They remained in the cytoplasm despite reconstitution of new nucleoli in daughter cells. The early sea urchin experiments were designed to duplicate the unique cobalt effect, thereby, hopefully, pinpointing critical stages during early development (Mateyko, 1961). The prominent nucleolus, a characteristic feature of the large germinal vesicle in the oocyte of *Arbacia* has always attracted attention. To illustrate, recently, Esper (1965) discussed the cytochemistry of its vacuole. But the information on nucleoli in blastomeres of echinoids is difficult to assess. In fact, it was only recently that the karyotype of *Arbacia punctulata* was published (Auclair, 1965). German (1964) demonstrated that of the 44 chromosomes, two pairs were considerably larger. Whether these are the nucleolar-organizer chromosomes or even whether nucleoli are organized into compact bodies associated with specific chromosomes in the early cleavage stages is not established. Cowden and Lehman (1963) demonstrated that there were no nucleoli in the blastula of *Lytechinus verigatus* (*sic*) or *Melita quinquesperforata* (*sic*). It was only at the early gastrula, 12 hours post-fertilization, that nucleoli became visibly organized. Is "blastulation" used in the sense of the onset of asynchrony, and this inception of differentiation a critical moment in nucleolar phe-

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nomena? Periodically, the view is reiterated that rapidly dividing cells (as in early cleavage of *Fundulus; vide,* Richards and Porter, 1935) are anucleolate, and organized nucleoli which appear at the blastula or gastrula stage are evidence of heightened synthetic processes in interphasic nuclei. It was thought that the induction of persistent nucleoli might be informative and thus cobalt glycine and cobalt chloride were tried. It was equally important to consider the possibility of reversing this effect with RNA or RN-ase, and since RNA synthesis dependent on DNA led to the study of the effects of DNA and DN-ase, the chain reaction extended as an evaluation of over a dozen substances at different dose levels and even more combinations (Mateyko, 1965). To avoid repetition and an unwieldy manuscript, the literature on control mechanisms and the levels at which metabolites affect the activities of macromolecules by specific interaction at the level of DNA, RNA, and protein synthesis appears in the discussion of the results.

Materials and Methods

The echinoderms, *Arbacia punctulata,* were dredged from the marine waters about Woods Hole, Massachusetts, during the summers of 1961, 1962 and 1963. Experiments were also repeated with *A. punctulata* obtained from the New York Aquarium through the courtesy of the Director, Dr. Ross Nigrelli. The sexes were separated (sex determined by electrical stimulation) and maintained in the laboratory in running sea water at a temperature of about 20° C. Healthy urchins moved about actively and voraciously ate *Laminaria.*

Ovulation and shedding of sperm were induced by electrical stimulation (Harvey, 1954). Viable eggs were fertilized by suspensions of sperm in sea water. Zygotes were placed in Syracuse dishes containing sea water and kept at 20° C. Observations were made at close intervals as to normal progress of development. The elevation of the fertilization membrane, patterns of cleavage, and rate and modification of growth were studied. Since the experiments were designed to test the effect of various substances on development *per se,* usually zygotes were used as the initial starting point. The fertilized eggs (incipient two-cell stage) were transferred to the experimental solutions in covered stender dishes. These were kept immersed in running sea water at a temperature of 20° C. for the duration of the experiment.

The substances, alone and in combination, were in solution and added to the sea water environments. The substances were: ethanol and methanol, reagent grade; cobalt chloride, C. P.; RNA (reagent grade), RN-ase (crystalline), DNA (sperm, not polymerized) DN-ase (70,000 Dornase units/mg.), sodium taurocholate, and coumarin from Nutritional Biochemicals Corp., Cleveland, Ohio; thymine, cobalt glycine, C. P., mescaline sulfate, papain (purified), and trypsin (1:300) from Mann Research Lab., New York, N. Y.; pronase (B grade), Calbiochem, Los Angeles, Cal.; actinomycin D provided through the courtesy of Merck, Sharp, and Dohme, Rahway, N. J.; thalidomide through the courtesy of Dr. Martin Kuna, Bristol-Myers, New York, N. Y.; 4-nitroquinoline N-oxide through the courtesy of Dr. P. O'B. Montgomery, Southwestern Texas Medical School, Dallas, Texas.

Components were added to the sea water in what approximated physiological levels; that is, the literature was consulted to determine what these levels were, if any, and pilot experiments were made to assess the physiological levels. Lethal, inhibitory, or ineffective concentrations of these agents were determined and appear as part of the observations. The effects of ethyl and methyl alcohol were also checked since these were used as solvents in some instances.

Each set of experiments involving a series of chemical agents (plus controls) utilized zygotes obtained from the same genetic background (one female, one male). Each set of experiments was repeated several times always using zygotes of uniform genetic background for the entire set of experiments to insure valid comparisons of development.

At close intervals the living embryos were examined, generally hourly, at a magnification of 100 × and compared with the controls as to stage of development.

In order to facilitate a true comparison of the degree of retardation, acceleration, or modification of development by environmental factors, a series of normal developmental stages were identified and given a code name. This stage series appears in the Results.

Photographs of the developing urchins under normal or modified environmental conditions were taken with a Zeiss Photomicroscope on Kodak Plus X 35 mm. film.

RESULTS

Stage series

Since it is well-known (Harvey, 1956) that temperature alterations may accelerate or retard development of *Arbacia,* all embryos in any experiment must be maintained at the same temperature throughout the duration of an experimental series. In order to facilitate a valid comparison of the degrees and types of retardation, acceleration, or modification of development brought about by various agents or environmental factors, a series of normal developmental stages at 20° C. has been identified by a code letter (Table I). Table I presents the usual sequential stages in the early development of *Arbacia punctulata.* In E. B. Harvey's (1956) book some of these have been charted, but the tabulation is not complete for any one temperature sequence.

The value of this series of stages is fourfold: (1) stages may be clearly identified by symbol; (2) precise stages are thereby stated (thus avoiding time factors which may lead to errors when environmental conditions such as temperature, crowding, etc., are present); (3) modifications in development brought about by experimental procedures are more readily interpreted when they can be compared with an unequivocal normal stage; and (4) more especially, brevity in citation.

When control and experimental embryos are raised at the same temperature within the normal range, and when the readings (that is, evaluation of developmental stages of both the controls and experimentals) are at the same time intervals, one needs only refer to the *stages* of development listed in Table I for a comparison of the progress of development. This is far preferable to expressing development in terms of *hours* of development, which often may be misleading because of the variability. No photographs of these stages are provided since Table I gives the description of each stage and photographs would be repetitious of the excellent and readily available Harvey (1956) illustrations.

All descriptions of development in subsequent tables are based on the stages listed in Table I, but amplification is made whenever abnormalities occur. In general the following tables list only a brief spectrum of pertinent data to avoid the wealth of detail.

Alcohol

Since many water-insoluble substances (for example, actinomycin D) are dissolved in alcohols, before an aliquot was added to the medium, effects of ethanol and methanol were rechecked on developing embryos (Table II). Both ethanol and methanol at a concentration of 1% or less appeared to have no visible morphologic effect on development beyond a slight retardation which was slightly greater in ethanol. At 5%, development proceeded only to the morula stage. The displacement of pigment to a compact mass was quite noticeable in 5% ethanol. At concentrations of ethanol greater than 5%, alcohol acted as a fixative immobilizing the two-cell stage. Development in both 5% and 10% methanol proceeded

TABLE I

Designation of early developmental stages in Arbacia punctulata

Stage designation	Morphological appearance in normal development	Time from insemination	
		T = 23° C.*	T = 20° C.
IE	Immature ovarian and non-fertilizable egg (germinal vesicle)	−6 h.	−6 h.
E	Mature, haploid, unfertilized egg	0	0
FE	Fertilized egg (fertilization membrane, hyaline layer)	2 m.	5 m.
S	Streak (two pronuclei have fused and centrosomes form curved streak)	20–35 m.	35 m.
2C	Two defined cells (first cleavage)	50 m.	60 m.
4C	Four defined cells (second cleavage)	78 m.	100 m.
8C	Eight defined cells (third cleavage)	103 m.	140 m.
12C	Twelve defined cells (beginning fourth cleavage)	130 m.	170 m.
16C	Sixteen defined cells (completed fourth cleavage)	135 m.	178 m.
32C	Thirty-two defined cells (fifth cleavage)	167 m.	210–215 m.
M	Morula (sixty-four cells, sixth cleavage)	4 h.	4½–5 h.
EB	Early blastula (seventh and eighth cleavages, asynchrony)	6 h.	7–8 h.
B	Mid-blastula (*ca.* 500 cells)	—	9 h.
H	Hatched blastula (*ca.* 1000 cells, free of fertilization membrane)	7–8 h.	9½–11 h.
SB	Swimming blastula	—	12–15 h.
EG	Early gastrula (beginning invagination)	12–15 h.	15–16 h.
G	Gastrula (gut complete, skeleton forming)		17–19 h.
LGP	Late gastrula or prism (change of larval axis from ovoid to polygonal)	18–19 h.	21–23 h.
P1	Pluteus larva (pentagonal)	20 h.	23–25 h.
P2	Pluteus larva (bulge of arms appears)	22 h.	26–29 h.
P3	Pluteus larva (well-defined pluteus)	24 h.	30 h. +
P4	Pluteus larva (Long-armed, two-days old)	48 h.	55 h.
P5	Pluteus larva (maximum development, long-armed, before feeding)	72 h.	80 h. +
ML 1	Metamorphosing larva (+ one pair new arms)	11 d. +	12 d. +
ML 2	Metamorphosing larva (+ additional pair of arms)	21 d. +	24 d. +

* According to E. B. Harvey (1956), p. 97
D = dead organisms.
EX = exogastrulation.

TABLE II

*The effects of ethanol and methanol on early development of A. punctulata**

Control Sea water	Experimental Sea water + alcohol							
All immersed as 2C	1% ETOH	5% ETOH	10% ETOH	25% ETOH	1% MEOH	5% MEOH	10% MEOH	25% MEOH
64C = M	M	M, pigment displaced	2C, D diffuse pigment	2C, D swollen	M	M	M, pigment displaced	2C, D fixed
LGP	LGP	M, D	—	—	LGP	M, D	M, D	—
P3	P2 sluggish	—	—	—	P2	—	—	—
P4	P3	—	—	—	P3	—	—	—

* Developmental stages at $t = 20°$ C. See Table I for descriptions.
D = dead embryos

only to the morula stage, with a clumping (dissolution) of echinochrome evident in 4 hours in 10% methanol. In the present experiments the levels of alcohol, where needed as a solvent (actinomycin D), were well below 1%.

Enzymes

One of the effects of trypsin, papain, pronase, and sodium taurocholate is on the cell surface. It is evident that trypsin attacks a cell-bonding substance, protein in nature, since disaggregation to blastomeres as well as dissolution of the fertilization membrane occurred in 4 hours. Development, however, did not proceed beyond the early blastula stage (Table III). In fact the blastomeres were digested.

TABLE III

*The effects of trypsin, papain, pronase, and sodium taurocholate on early development of A. punctulata**

Control Sea water	Experimental Sea water +						
						Na taurocholate	
All immersed as 2C	Trypsin 1 mg./ml.	Trypsin 2 mg./ml.	Papain 0.5 mg./ml.	Papain 1 mg./ml.	Pronase 2.5 mg./ml.	0.2 mg./ml.	2 mg./ml.
8C	8C	8C	8C	4C	8C[1]	8C	4C
M(64C)	M[1]	M[1]	M	8C	M	M	M
LGP	EB	EB, D	EB[2]	M, D	LGP	LGP	EB, D
P1	D				P1	P1	
P3			H, D		P3	P3	
P4					P4	P4	
P5					P5	P5	

* Developmental stages at $t = 20°$ C. See Table I for descriptions.
D = dead embryos.
[1] Fertilization membrane digested and disaggregation to blastomeres.
[2] Slight separation of blastomeres.

To maintain the blastomeres, the embryo must be removed to fresh sea water. They, however, did not reaggregate. Papain exerts a more drastic direct effect even at a concentration of 0.5 mg./ml., in that only a few zygotes become morulae, and still fewer reach the blastula. Separation to blastomeres occurred but was not especially striking. The fertilization membrane is more resistant here than in trypsin. Sperm remained motile in pronase (0.25 mg./ml.) even after two hours, but the motility was reduced by three hours. Eggs, both unfertilized and fertilized, exhibited a striking reduction in cortical consistency. The response of the fertilization membrane to pronase was not clearcut, for while 5% of the fertilized eggs had a distinct membrane, the remainder did not. It may, therefore, be inhibited or lysed. Lysis of a developed fertilization membrane occurred about 2 hours later. Development proceeded without the membrane. Separation of blastomeres occurred at 8C or 16C. The lack of a fertilization membrane obscured the decision as to whether these cells can divide still further. Sodium taurocholate in a low concentration (0.2 mg./ml.) did not appear to damage the cells or retard development but at a concentration of 2 mg./ml. development ceased at early blastula, with swelling of the blastomeres and surface erosion. Survival in all instances was enhanced by washing at any time before lethal effects had set in.

TABLE IV

*The effects of DNA on early development of A. punctulata**

Control	Experimental			
Sea water	DNA			
All immersed as 2C	DNA 0.01 mg./ml.	DNA 0.02 mg./ml.	DNA 0.05 mg./ml.	DNA 0.1 mg./ml.
B				M
H	H		B	H
EG				EG
G	EG			
LGP	G, LGP	G, LGP	LGP	G, LGP
P1			P1	
P2	P1	P1	P2+	LGP, sluggish
P3	P2	P2	P2	LGP, D
P4	P3	P3	P3	
P5	P4	P4	P5	

* Developmental stages at $t = 20°$ C. See Table I for descriptions.
D = dead embryos.

DNA

At a concentration of 0.5 mg./ml. of DNA, development throughout is similar to that of the controls, for the embryos achieve the maximum development possible without the addition of food. At double the concentration, retardation in growth is striking and many specimens die. Skeletal formation continues in organisms which still maintain their original ovoid (gastrular) shape, often within a persistent fertilization membrane. The effect is reversible if specimens are removed and washed after 9 hours. Lower amounts of DNA, 0.01 to 0.02 mg./ml. do not alter appreciably the morphogenetic patterns or rate of development (Table IV).

DN-ase

Dn-ase added to sea water effects a consistent developmental pattern. At 4 μg./ml. the rate of development appears very slightly higher than in the controls (Table V). At 8 μg./ml. the effect is not detectable morphologically—the specimens look like the controls. In 0.05 mg./ml. the activity of the animals is curtailed—the movements are slower, while the rate of development is unaltered. Retardation of growth and activity is clear in 0.1 mg./ml. and more pronounced in 0.2 mg./ml., the animals dying in 48 hours. The morphology, however, is normal. The retarding effects can be reversed by removing the embryos from the medium even after 9 hours of development in 0.1 mg./ml. DN-ase.

TABLE V

*The effects of DN-ase on early development of A. punctulata**

Control Sea water	Experimental Sea water + DN-ase				
All immersed as FE	4γ/ml.	8γ/ml.	0.05 mg./ml.	0.1 mg./ml.	0.2 mg./ml.
Developmental stages					
B				B	
H		H			
SB	SB		SB, slow		
EG		EG		EG	
G				G	EB
LGP	LGP, P1	G, LGP	LGP	G, LGP	EG
P1	P2		P1		
P2		P1		P1	LGP
P3	P3+	P3	P3	P2	P1
P4	P4+	P4	P4	P3	P2
P5	P5+		P5	D	D

* Developmental stages at t = 20 °C. See Table I for descriptions.
D = dead embryos.

RNA

RNA was made up as a stock solution of 0.4 mg./ml. Stronger solutions (1 mg./ml.) cannot readily be made up in distilled water since the solubility is low and precipitation occurs. Solubilization by other means is likewise undesirable since it leads to degradation of the molecule.

The effects of RNA depend upon the dosage. At 0.1 mg./ml. and greater, retardation of growth occurs which is especially noticeable at gastrulation. Development through blastulation is, however, normal and no anomalies occur (Table VI). In 0.04 mg./ml. RNA, a slight acceleration of development up to gastrulation is evident, but it slows down subsequently to less than normal. Thus at lower concentrations of RNA the effect is one of enhancement of development. With a medium containing 16 to 20 μg./ml. the effect is imperceptible morphologically, for development proceeds at a normal pace and the embryos are in excellent condition at all stages. With 10 μg./ml., the early acceleration of development is most

TABLE VI

*The developmental effects of RNA on early development of A. punctulata**

Control Sea water	Experimental RNA						
All immersed as 2C	RNA 4 μg./ml.	RNA 8 μg./ml.	RNA 10 μg./ml.	RNA 16 μg./ml.	RNA 20 μg./ml.	RNA 0.04 mg./ml.	RNA 0.1 mg./ml.
B	B	B			B		B
H			EG			G	
SB	SB	SB	G		SB		SB
LGP	G	G, LGP		G		G, LGP	G
P1			P2		P1		LGP
P2			P2		P2	P1	P1
P3	P3	P2	P3	P2	P3	P2	P2
P4		P3	P4	P4	P4	P3	P2
P5	P5	P5					

* Developmental stages at $t = 20°$ C. See Table I for descriptions.

clear—the embryos gastrulate in advance of the control specimens. By 24 hours and subsequently, the spurt in development is less evident. Thus, it is questionable whether augmentation of sea water with 4 to 8 μg./ml. of RNA is effective in bringing embryos earlier to metamorphosis (Table VI). Washing the embryos free of the medium after development for several hours (5 to 9 hours) does not reduce the effect.

RN-ase

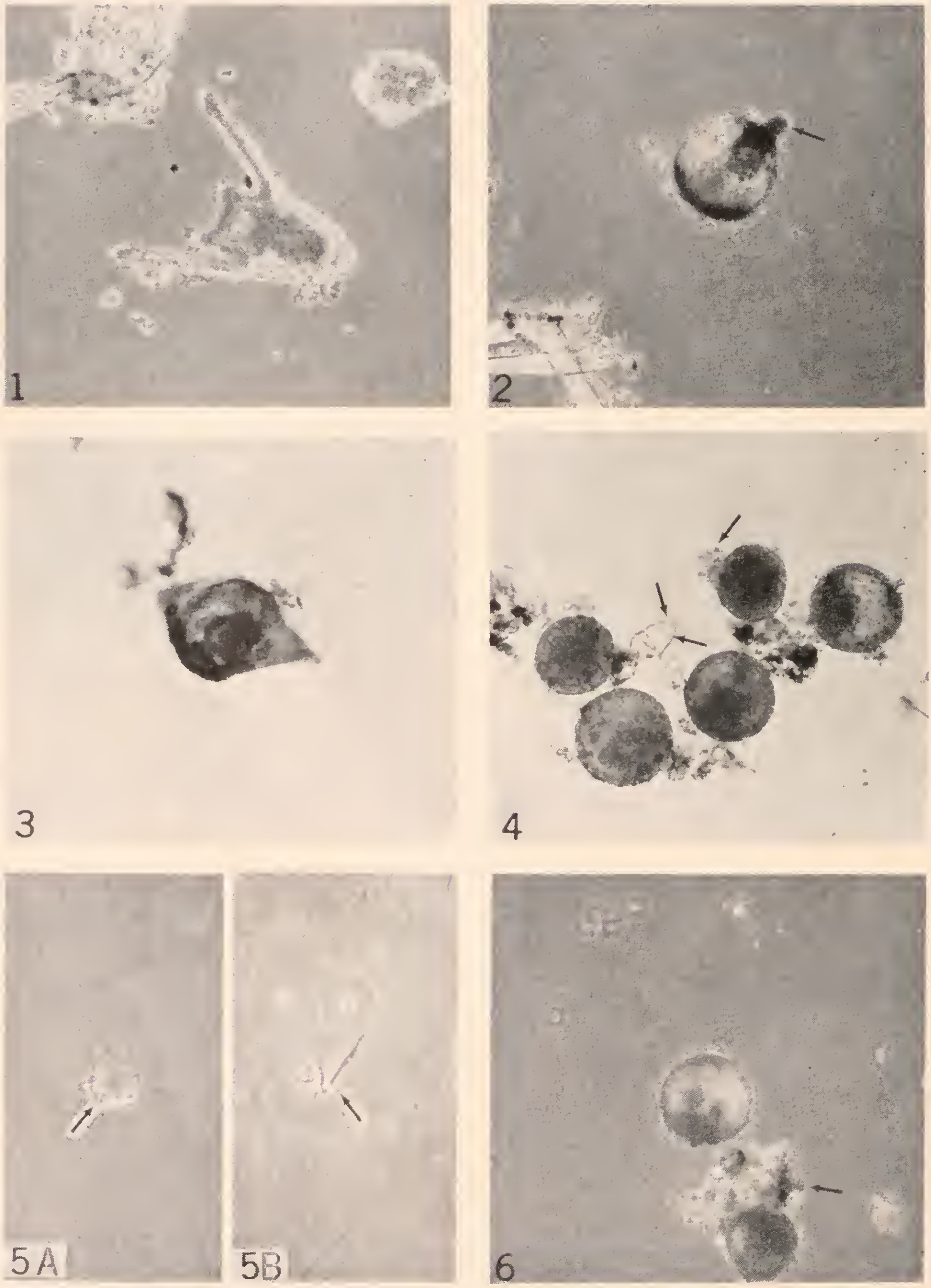
Unlike RNA, RN-ase is readily soluble. Stock solutions of 1 mg./ml. and 1.2 mg./ml. were prepared with distilled water.

TABLE VII

*The effects of RN-ase on early development of A. punctulata**

Control Sea water	Experimental Sea water + RN-ase					
All immersed as 2C	RN-ase 1.2 μg./ml.	RN-ase 12 μg./ml.	RN-ase 0.04 mg./ml.	RN-ase 0.05 mg./ml.	RN-ase 0.1 mg./ml.	RN-ase 0.12 mg./ml.
B	B				B	
H				H	H	H
SB	SB			SB	SB	SB
EG					EG	EG
G	G			G	G	
LGP	LGP	LGP	LGP	LGP	LGP	G, LGP
P1	P1			P1		G, LGP
P2						P1
P3	P3+	P3	P3	P3	P2	P2
P4	P4	P4	P4	P4	P3	P3
P5	P5		P5	P5	P4	P4

* Developmental stages at $t = 20°$ C. See Table I for descriptions.



Figures 1–6.

In RN-ase, the developmental effects follow a consistent pattern. With 1.2 μg./ml., to 0.05 mg./ml. an effect is lacking in that the embryos develop at a normal rate and appearance (Table VII). At 0.1 to 0.12 mg./ml. a slight retardation in the rate of growth is clear, the stages through gastrulation following a normal pattern. Embryos, however, are sluggish. Despite this, the inhibition is reversible if embryos are put into fresh sea water even after 9 hours development. No developmental anomalies were seen. Figure 1 illustrates a typical normal-appearing, long-armed pluteus (48 h.) raised in 0.1 mg./ml. RN-ase for 9 hours and then placed into fresh sea water.

Thymine

In low concentrations of thymine (0.04 mg./ml.) development proceeds normally but at a slower rate as if raised at a lower temperature. In 0.1 mg./ml. the retardation is exaggerated, and in 0.2 mg./ml. the retardation is even more drastic, and with 0.4 mg./ml. most of the embryos succumb at about blastulation (Table VIII). Malformations are rare. Gastrulation is noticeably interfered with at 0.4 mg./ml. since the embryos are moribund at this stage and are dead within 24 hours. The addition of RN-ase (0.5 mg./ml.) to thymine (0.1 mg./ml.) treated embryos does not significantly alter retardation brought about by thymine. With the addition of DN-ase (10 μg./ml.) to thymine (0.1 mg./ml.), the retardation which occurs is more evident after pluteus formation, since the well-formed plutei were small. Development through blastulation is normal in DNA (0.05 mg./ml.) plus thymine (0.1 mg./ml.) but an arrest occurs at the prism stage. Despite a persistent fertilization membrane, skeleton formation proceeds, but subsequently the embryos die. If embryos are washed free of the thymine, recovery is rapid.

Cobalt

An analysis of Table IX will show that in below lethal levels, the striking effect of cobalt chloride and cobalt glycine is the production of exogastrulae (Fig. 2). Where lethal effects were evident at stage 2C to 16C these were not tabulated. In general, the effect of both compounds is similar at a concentration of 0.02 mg./ml. If ova are fertilized in sea water containing cobalt salts, the effect arises sooner than if the 2C stage is used at the starting point.

FIGURE 1. *Arbacia punctulata* embryo at 48 hours; in RN-ase (0.1 mg./ml.) for 9 hours, washed; note excellent development of long-armed pluteus; phase, 145 ×.

FIGURE 2. Embryos reared in cobalt chloride (0.02 mg./ml.) for 3 days. Exogastrulation evident (arrow) and further differentiation inhibited; phase, 145 ×.

FIGURE 3. "Pollen grain" form induced by cobalt glycine (0.02 mg./ml.) after 3 days; 145 ×.

FIGURE 4. Note irregular alignment of cells (arrow) and pronounced exogastrulation (between arrows) in embryos raised in cobalt glycine (0.01 mg./ml.) for 48 hours. Change to pluteus form is inhibited; 145 ×.

FIGURE 5. Isolated spicules are reduced in size and deformed. 5A: cobalt glycine (0.02 mg./ml.) 48 hours. 5B: cobalt chloride (0.02 mg./ml.) 48 hours; phase, 180 ×.

FIGURE 6. Two-day-old embryo (cobalt chloride = 0.01 mg./ml.). Note large exogastrulate mass (arrow) with poor alignment of cells. Mass of cells emerges from spherical gastrula; phase, 145 ×.

Development in cobalt proceeds normally through the blastula although it is slowed down. In minimal levels (< 0.01 mg./ml.) embryos exhibit a growth retardation with sluggishly and erratically swimming forms. At higher concentrations (0.01 to 0.02 mg./ml.) the drastic exogastrulation produces such abnormal embryos ("pollen grain" forms) that normal plutei and metamorphosis cannot occur. Exogastrulation is preceded by a bloating which persists and results in a form that resembles a winged pollen grain (Fig. 3). In some (cobalt glycine, 0.01 mg./ml.) the surface is rough, owing to a lack of alignment of surface cells (Fig. 4). In all, however, spicule formation does occur. The skeleton is reduced to deformed microspicules in 0.01 mg./ml. cobalt glycine (Fig. 5a and b). Some malformations are so bizarre (cobalt glycine 0.02 mg./ml.) that a picture is preferable to a description (Fig. 6). The "pollen grains" spin around in eccentric orbits unlike the smooth swimming of normal plutei. The developmental arrest was reversible if the embryos were washed repeatedly (after growth in cobalt for 5 hours). At first recovery was slow, but within 48 hours, the embryos appeared similar to normal. Once grossly malformed embryos ("pollen grains") develop and gastrulate, a return to a normal post-gastrulation pattern cannot occur. The addition of DNA, or DN-ase could not inhibit or reverse the cobalt effect to any useful degree (Table IX) even though RNA (10 μg./ml.) and RN-ase (0.05 mg./ml.) slightly ameliorated the effect.

TABLE VIII

*The effects of thymine on early development of A. punctulata**

Control Sea water	Experimental Sea water + Thymine				Thymine +		
All immersed as FE	0.04 mg./ml.	0.1 mg./ml.	0.2 mg./ml.	0.4 mg./ml.	0.1 mg./ml. +0.05 mg./ml. RN-ase	0.1 mg./ml. +0.05 mg./ml. DNA	0.1 mg./ml. +10 γ/ml. DN-ase
16C	4C			4C			
B			B				
H	EB			B, slow	B	H	H
G		EG	G, slow		H		
P1	LGP	LGP	LGP	G, LGP moribund	LGP	LGP	LGP
P2		P1	P1				
P3	P3		P2	D	P2	LGP, D f.m. present	P2, small
P4	P4	P2	P3		P3		P2
P5	P5 sluggish	P4	D				

* Developmental stages at $t = 20°$ C. See Table I for descriptions.
D = dead embryos.
f. m. = fertilization membrane.

Actinomycin D

The results of growth in actinomycin D with or without other compounds are summarized in Table X. In general, all dose levels, even as low as 3 μg./ml.,

TABLE IX

*The effects of cobalt chloride and cobalt glycine on early development of A. punctulata**

Part A

Control Sea water	Experimental Sea water +					
	Cobalt chloride				Cobalt glycine	
Developmental stages	0.02 mg./ml.	0.02 mg./ml.	0.02 mg./ml. +4 γ/ml. DN-ase	0.02 mg./ml. +0.5 mg./ml. RN-ase	0.01 mg./ml.	0.01 mg./ml.
Immersed as E+S	E+S	2C	E+S	E+S	E+S	2C
32C						4C
EB	EB	EB			M	M
H	H	H			EG	EG, H
EG				M		
G	SB					
LGP		LGP	G, LGP	B		
P1	EG	LGP swollen	G, EX	EX	G	G swollen
P2	EX	EX	EX sluggish	EX active		LGP
P3	EX	EX sluggish			G, LGP malformed	LGP, sluggish malformed
P5		EX, highly malformed, spin	EX, moribund	P1 malformed		LGP, malformed

Part B

Control Sea water	Experimental Sea water +					
	Cobalt glycine			Cobalt glycine +		
Developmental stages	0.02 mg./ml.	0.2 mg./ml.	1 mg./ml.	0.01 mg./ml. +0.05 mg./ml. DNA	0.02 mg./ml. +8 γ/ml. RNA	0.02 mg./ml. +4 γ/ml. DN-ase
Immersed as E+S	2C	2C	2C	2C	2C	2C
32C		2C, 4C	4C	4C	4C	4C
EB					M	M
H		M	M, D		EG	
EG				M	H	H
G						
LGP	EX	M, B		B	LGP sluggish	G, LGP
P1	LGP	B, M, D		G bloated	LGP malformed	LGP malformed
P2	LGP, EX malformed			EX swollen	LGP malformed	LGP malformed
P3						
P5	LGP malformed			EX swollen	LGP malformed	

* Developmental stages at $t = 20°$ C. See Table I for descriptions.
D = dead.

TABLE X

*The effects of actinomycin D on early development of A. punctulata**

Part A

Control Sea water	Experimental Sea water + actinomycin D							
Immersed as FE	AD 3 μg./ml.	AD 4 μg./ml.	AD 8 μg./ml.	AD 20 μg./ml.	AD 25 μg./ml.	AD 30 μg./ml.	AD 0.75 μg./ml. +5 μg./ml. DNA	AD 4 μg./ml. +0.01 mg./ml. DNA
32C					2C, 4C			
B	B	EB, "lumpy"	EB "lumpy"	M	8C	4C		
H								
SB		B						
LGP	B, G	EG	H, slow		M, D	M, D	EG	G, slow
P1		LGP	G, cell separation					
P2	LGP	LGP, bloated	G, D	M, D				G, moribund
P3	P1	P1, bloated					G	
P4		P1, bloated					LGP	LGP, moribund
P5		P1, have f.m. no arms					D (75 h.) no arms	No skeleton

Part B

Control Sea water	Experimental Sea water + actinomycin D$^+$							
Immersed as FE	AD 8 μg./ml. +0.02 mg./ml. DNA	AD 4 μg./ml. +4 μg./ml. DN-ase	AD 8 μg./ml. +0.1 mg./ml. DN-ase	AD 4 μg./ml. +4 μg./ml. RNA	AD 4 μg./ml. +20 μg./ml. RNA	AD 8 μg./ml. +8 μg./ml. RNA	AD 4 μg./ml. +4 μg./ml. DN-ase	AD 4 μg./ml. +0.05 mg./ml. thymine
32C								
B							B	B
H		M					H	H
SB							SB	SB
LGP	8C, D	B	M, separation of cells	G	SB	M, D	LGP moribund	LGP swollen
P1			M, D		LGP			P1
P2		G, malformed		G, LGP	LPG, swollen			
P3				LGP	LGP, no arms			
P4					LGP			
P5								

* Developmental stages at $t = 20°$ C. See Table I for descriptions.
D = dead embryos.
f.m. = fertilization membrane.

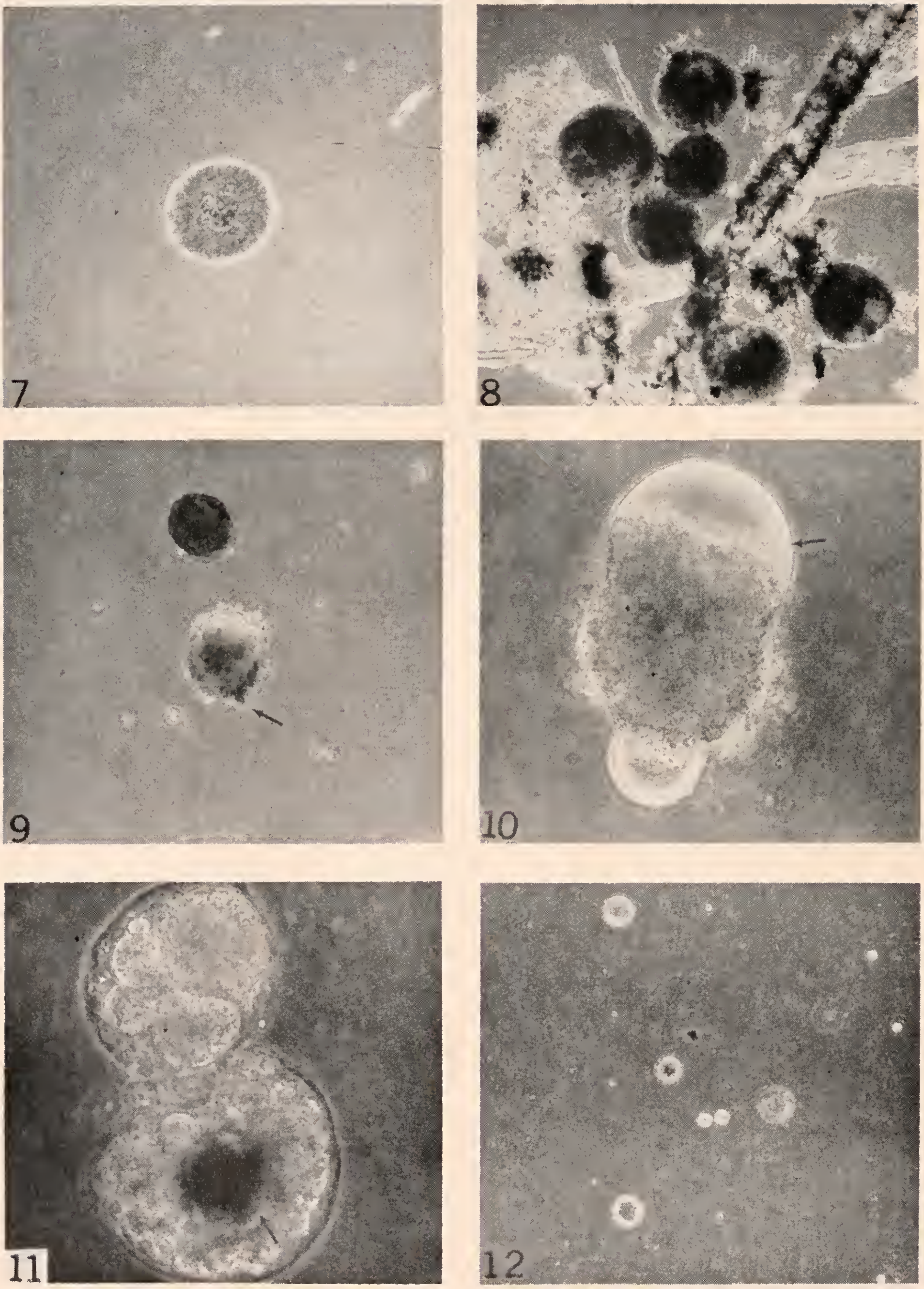
exerted a noticeably retarding effect on development and the retardation was dose-dependent. At 4 μg./ml. the retardation is minimal in the early stages and more striking post-gastrulation in that poorly developed gastrulae cannot differentiate into the pluteus stage. Figure 7 represents a surviving 2-day-old "gastrula" (4 μg./ml.). The bloated larvae (Fig. 8) never develop a skeleton and arms do not develop even after 80 hours survival. With higher concentrations, 8 μg./ml., 20 μg./ml., and 25 μg./ml., the arrest is striking, showing gastrular anomalies (8 μg./ml.), and at 20 μg./ml., 25 μg./ml., and 30 μg./ml., development does not proceed beyond the morula. At 40 μg./ml. most die before the morulae. Doses higher than 40 μg./ml. were not tried because a marginal sensitivity occurred at 2 μg./ml., and a concentration as high as 0.15 mg./ml. existed as a suspension.

The effects are reversible in so far as survival is concerned, by washing at least within 15 hours, in concentrations up to 8 μg./ml. After that period, washing does not enhance survival. At concentrations of 15 μg./ml. and higher the effects do not appear to be reversible and the embryos die. DN-ase (0.1 mg./ml., 4 μg./ml.) and RNA (4 μg./ml., 8 μg./ml., and 20 μg./ml.) likewise were ineffective in reversing the actinomycin D effect (4 μg./ml., 8 μg./ml.). While RN-ase (0.05 mg./ml.) and thymine (0.05 mg./ml.) appear to ameliorate slightly the actinomycin D (4 μg./ml.) effect in early cleavage stages, the post-gastrulation stages were still retarded. DNA in low concentrations (5 μg./ml. to 0.01 mg./ml.) does not detectably counteract the actinomycin D effect, but at 0.2 mg./ml. does appear to lessen the drastic actinomycin D effect, especially since survival through the pluteus stages occurred. Oddly enough, 0.05 mg./ml. thymine likewise enhanced survival through a young pluteus stage, but again, no arms were formed. The inhibition of formation of arms and skeleton in the pluteus was a characteristic feature even up to three days of development.

The developmental effects were similar whether eggs and sperm were fertilized in actinomycin D solution or the zygotes were placed into it, and embryos did not show subsequent developmental divergence. If embryos were washed within 9 hours of immersion (4 μg./ml.) they appeared in a better condition than the ones that were unwashed, but the difficulties experienced in gastrulation (abnormalities such as lack of skeleton and arms) were still evident. At 4 μg./ml. there was some evidence of a persistent fertilization membrane about abnormal embryos as late as 80 hours of development. Besides the slowing down of early cleavages, actinomycin D appears to specifically inhibit differentiation of arms and the skeleton. This is especially striking after 75 hours of growth where malformed, arrested late gastrulae eventually die. Figure 8 illustrates these gastrulae, which, while alive, are poorly differentiated, lacking arms, skeleton, and other features of comparable plutei of this age. Another observation bears mention: that is, blastomeres appear to separate at gastrulation with loose cells in the blastocoele (8 μg./ml.). The cleavage disorganization appears quite early, even before the formation of the morula, in that rather "lumpy" or irregularly surfaced embryos develop. This is evident even at a concentration of 4 μg./ml.

4-nitroquinoline-N-oxide

The compound, 4 nitroquinoline-N-oxide (4 NQ), was quite toxic. Eggs were fertilized by normal sperm (presence of fertilization membrane) and immersed in



FIGURES 7–12.

a concentration of 0.125 mg./ml., but no cleavage ensued. The cytoplasm of the zygote was shrunken away from the surface of the egg (Fig. 10). Sperm, however, were quite viable in this concentration, showing vigorous activity even after $4\frac{1}{2}$ hours. In 0.0125 mg./ml. an irregular cleavage proceeded along up to the morula stage, with a concomitant separation of blastomeres. Pigment condensation likewise occurred. At half this level, development proceeded to the blastula stage, where it was arrested. Figure 11 illustrates two embryos with irregular-sized blastomeres and pronounced pigment condensation in one. In Figure 12 evidence of complete dispersion of blastomeres is seen after 24 hours.

Coumarin

In coumarin (0.5 mg./ml.) development was slower than normal and never beyond the morula stage. The retardation was more effective if eggs and sperm were immersed separately than after fertilization. If the embryos were washed after five hours development in coumarin (at M stage) some survived for 30 hours. Most of these embryos, however, were deformed spherical masses of cells and spun in eccentric orbits. Figure 13 illustrates embryos in a coumarin solution (0.5 mg./ml.) after 46 hours. Some of the embryos have disaggregated to isolated cells and cell clusters; others are highly irregular but their viability is attested to by their active spinning. By 48 hours most were dead and separation of blastomeres ensued. No gastrulation occurred and no skeletal formation was evident. The block at the blastula stage was striking and irreversible.

Mescaline

A concentration of 1 mg./ml. is lethal, and organisms generally die about gastrulation. Most succumb at the B and M stage. In 0.1 mg./ml. development proceeds but the rate is slower than normal. While the formation of arms in plutei is inhibited, a minuscule skeleton (one-sixth normal size) forms. Although the specimens in Figure 14 appear to be "prisms," they are actually 48 hours old with arm development strikingly inhibited.

RNA (4 μg./ml.) added to the (0.1 mg./ml.) mescaline medium brings about a sharp arrest at gastrulation. Abnormal cell masses due to irregular cleavage result. The 48-hour "plutei" die but bear small spicules. On the other hand, the substitution of DNA (0.05 mg./ml.) terminates development at the blastula stage which developmentally coincides with the prism stage of the control (Table

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FIGURE 7. Arrested embryo in 4 μg./ml. actinomycin D for 48 hours. Note inhibition of gastrulation; phase, 145 ×.

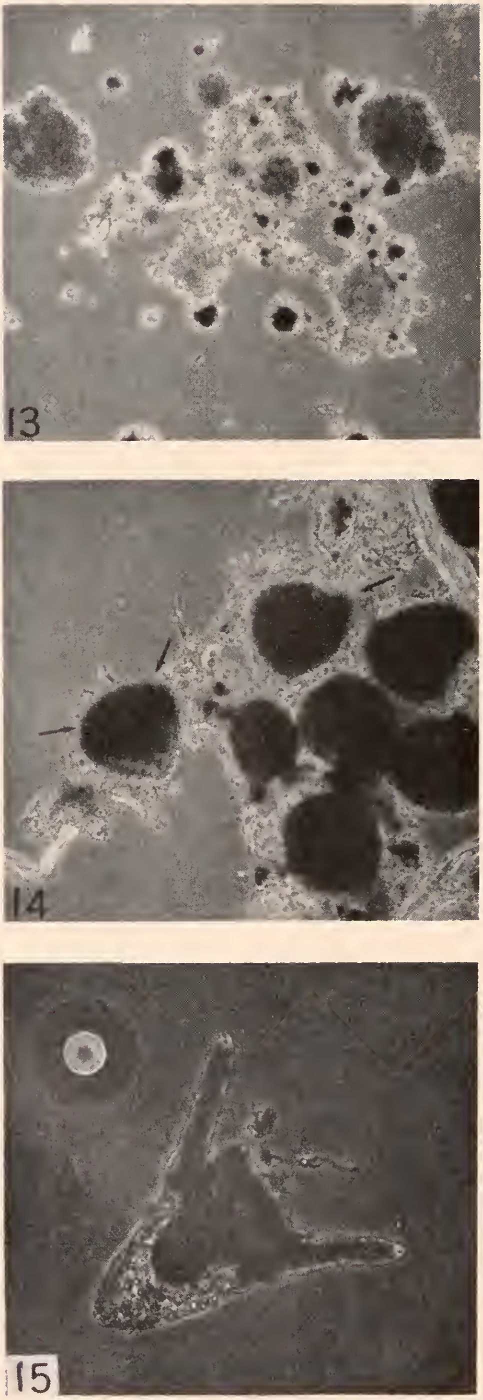
FIGURE 8. Surviving embryos in 4 μg./ml. actinomycin D for 80 hours. Differentiation is halted at gastrulation; phase, 145 ×.

FIGURE 9. With higher levels of actinomycin D (8 μg./ml.) embryos show pronounced irregularity in deposition of surface cells (arrow) and gastrulation is abnormal; phase, 145 ×.

FIGURE 10. Egg of *Arbacia* in 0.125 mg./ml. 4-nitroquinoline N-oxide with drastic shrinkage of cytoplasm (arrow) and cytolysis evident in 15 minutes; phase, 360 ×.

FIGURE 11. Larvae in 12.5 μg./ml. 4-NQ after 8 hours. Note irregular size of blastomeres and pigment condensation (arrow); phase, 360 ×.

FIGURE 12. Complete dispersion of blastomeres after 24 hours in 12.5 μg./ml. 4-NQ; phase, 145 ×.



Figures 13–15.

XI). The effects of mescaline are not reversed by placing the embryos in fresh sea water if development has proceeded past gastrulation.

Thalidomide

The effects of thalidomide are charted in Table XII. Because of the poor solubility of thalidomide in aqueous solutions, the concentrations cannot be considered as absolute, but solution in other agents was contraindicated since chemical modifications of the molecule occur. At a level of 0.02 mg./ml. development followed normally; at 0.08 mg./ml. a slowing-down became evident; at 0.1 mg./ml. the retardation was clear. The slower development, nonetheless, did proceed successfully through gastrulation and up to the pluteus stage with some deformations. Figure 15 shows a 46-hour pluteus raised in 0.1 mg./ml. thalidomide. The arms are slightly shorter than those of the normal 2-day-old embryos, and the skeletal formation is poor. Just about the time when elongation of the arms of the pluteus occurred (stage P4–P5), the sluggish plutei died, many remaining within an intact fertilization membrane. At 1 mg./ml. the same pattern evolved but the retardation was greater. Of considerable interest is the persistence of

Table XI

*The effects of mescaline on early development of A. punctulata**

Control Sea water	Experimental Sea water + mescaline			
All immersed as FE	0.1 mg./ml.	1 mg./ml.	0.1 mg./ml. +4 γ/ml. RNA	0.1 mg./ml. +0.05 mg./ml. DNA
Developmental stages				
16C	8C	4C		
M	16C			
H				H
SB	H	M		
EG	SB			
G	EG	EB sluggish	G, with abnormal cell masses	D, as B
LGP	G			
P1	LGP, many dead	G	G, static	
P3	P1, sluggish			
P4	P1, no arms but skeleton		D, as G have skeleton, malformed	

* Developmental stages at $t = 20°$ C. See Table I for descriptions.
D = dead embryos.

Figure 13. Embryos although alive in coumarin (0.5 mg./ml.) after 48 hours consist of misshapen balls of cells with much separation of blastomeres; phase, 145 ×.

Figure 14. Specimens raised 48 hours in mescaline (0.1 mg./ml.). Arm development (arrows) arrested; phase, 145 ×.

Figure 15. A pluteus larva after 48 hours in thalidomide (0.1 mg./ml.). Morphology essentially normal except for slightly shorter arms and poorer skeletal formation than the control; phase, 180 ×.

TABLE XII

*The effects of thalidomide on early development of A. punctulata**

Control Sea water	Experimental Sea water + thalidomide					
All immersed as 2C	0.02 mg./ml.	0.1 mg./ml.	0.5 mg./ml.	1 mg./ml.	2.5 mg./ml.	0.5 mg./ml. +0.04 mg./ml. RNA
Developmental stages						
16C		4C, 8C		2C, 4C		
EB						
SB		M	EB	M		
G	G				32C	EG
LGP		G	EG		D	
P1	P1		LGP	G		
P2		P1		EG		
P3	P2, P3		P1	M, G		LGP, P1
P4	P4	P2, moribund	P3	LGP		P1, P2 sluggish
P5+	P5+	P3, D	P4, small	P1, inactive		

Control Sea water	Experimental Sea water + thalidomide				
All immersed as 2C	1 mg./ml. +0.04 mg./ml. RNA	1 mg./ml. +0.05 mg./ml. DNA	1 mg./ml. +10 μg./ml. DN-ase	1 mg./ml. +0.05 mg./ml. DNA +10 μg./ml. RNA	0.5 mg./ml. +0.1 mg./ml. cobalt glycine
Developmental stages					
16C					
EB		4C, D	M, D	2C, D	
SB					
G					
LGP	EG				SB, LGP
P1					
P2					[illegible]
P3	G, D				SB, LGP moribund
P4					P1
P5+					D

* Developmental stages at $t = 20°$ C. See Table I for descriptions.
D = dead.

the fertilization membrane about the plutei, which indicates a fault in the hatching enzyme. Greater doses were lethal (Table XII).

The thalidomide effect is irreversible in that even if the zygotes are washed free from the compound, the retardation persists. Moreover, it appears that the zygote is more adversely affected than if eggs and sperm are immersed separately prior to fertilization. Skeletal formation generally occurred normally. The addition of other agents (RNA, cobalt glycine, DNA, DN-ase, DNA + RNA) greatly enhanced the retardation or lethal effects of thalidomide.

DISCUSSION

General

The sea urchin egg and zygote, especially of the genus *Arbacia,* have been for many years the favorite material for the investigation of early morphogenesis. Within the last decade biochemical studies on the metabolism of nucleic acids, proteins, and carbohydrates have increased enormously. Since competent reviews and papers in various areas are available (*vide:* Gustafson, 1954; Costello, 1955; Ranzi, 1957, 1962; Deuchar, 1962; Guidice *et al.,* 1962; Karnofsky and Simmel, 1963; Gross, 1964; Lallier, 1964a; Monroy and Maggio, 1964, Brachet, 1965a, 1965b; *inter alii*), only particularly relevant papers will be cited without attempting to identify *seriatim* all the excellent work in this field.

The problem of comparing data not only from one experiment to another, but from one investigation to another has become a very real one. Comparisons are hindered because of variations during the experiments *per se.* As Harvey (1956) states: "There is a slight variation in cleavage times in different batches of eggs and considerable variation at different times of year, irrespective of temperature . . . there is a greater variation in the later stages of development, *e.g.,* time of hatching" (p. 97). Thus expressing development as being "5½ hours post-fertilization" or "20 hours development" as is often done, is uninformative. It is the precise developmental *stage* which needs to be identified, the stage at which critical and profound biochemical events come into play.

Accordingly, the symbols and developmental stages listed in Table I have been selected as being the most diagnostic—from the immature oocyte to the long-armed, non-feeding pluteus. The need for clarity, brevity, and accuracy in staging has been considered, as well as time and temperature factors. Although this series (Table I) refers specifically to *Arbacia punctulata,* the designated *stages* may be used as a guide for level of development in other species or genera in the echinoids, especially when data are compared. Hopefully, similar studies will be made on pre- and post-metamorphic echinoderms in order to establish and tabulate morphological criteria of the stage of development. The same cautions would apply as in the formulation of Table I.

The prime advantage of the present multi-pronged approach, that is, being able to compare the effects of a wide spectrum of agents, is that all departures from the normal developmental pattern can be readily compared and non-specific effects can be assessed as well as highly specific effects. The present morphological study may serve to supplement previous studies. Attempts were made to follow development through to the feeding pluteus larva, the embryo being considered as a total system.

Alcohol

Since actinomycin D (0.5 μg./ml.) had to be solubilized in 95% ethanol, the question of the effect of a weak alcohol solution on growth had to be rechecked. Over 50 years ago Lillie (1914) reported that 5% ethanol (0.87 M) acted as an anaesthetic on sea urchins in that there was a reversible arrest of cleavage. Later Blumenthal (1928) confirmed the anaesthetic effects of alcohol on sea urchin zygotes. Waterman's (1936) data indicated that ethyl alcohol up to 0.9% had no effect and retardation occurred with a concentration of 1 to 2%. A reversible effect was possible with concentrations up to 4.4% but 5% was lethal. Exogastrulae developed when concentrations ranged from 0.9 to 1.3%, but this was not observed in our studies.

The concentration of alcohol in the present experiments (actinomycin) was always well below 0.9% and, therefore, did not affect cleavage. Our experiments, likewise, confirmed the lethal effect of 5% ethanol and indicated that the effects of methanol (5%) were similar. The lethal higher concentrations of alcohol probably operate as lipid solvents and denaturating agents. The swelling, doubling in volume, in ethanol and methanol reported by Stewart (1931), was not evident.

Enzymes

Enzymatic dissociation of multicellular tissues is by now a routine procedure but it was deemed necessary to evaluate the effects of the commonly used proteolytic agents on sea urchin embryogenesis, *per se*.

Northrup (1947) demonstrated the fact that proteolytic enzymes (0.1 to 1 mg./ml.) did not lyse mature protein in that *Arbacia* developed normally in ficin and trypsin, with separation of blastomeres occurring only in papain. He emphasized the fact that trypsin did not affect living cells with native protein and attacked only denatured protein. Moore (1949), on the other hand, observed trypsin lysing the fertilization membrane and effecting cell disaggregation. Later, Moore (1952) followed the gastrulation of *Dendraster* in trypsin (1 mg./ml.). Embryos with elevated fertilization membranes showed a lysis of this structure within 5 to 10 minutes. The denuded embryos generally lost blastomeres at invagination, thus larvae developed as malformed plutei with defective skeletons. Moore's (1951) interpretation was that blastomeres released tryptic inhibitors.

The present results confirm the usefulness of trypsin as a dispersing agent for blastomeres and fertilization membranes. It must be used at less than 0.5 mg./ml. and be washed free from the cultures within one hour and certainly before 4 hours, to prevent death of the embryos. The harmful effects may be due to the penetration of trypsin into cells as has been shown by Weiss (1958), who determined that a 20% loss in the dry mass of sarcoma 37 cells occurred as a result of trypsin treatment.

The data of Jensen (1950) and Hörstadius (1953) indicate that ficin and trypsin have similar proteolytic activity. The action of papain is somewhat similar. In 1947 Northrup described the separation of *Arbacia* blastomeres in papain. We confirmed this observation and also observed the dissolution of the fertilization membrane. The lysis, however, took longer than in trypsin and undesirable cytologic effects also resulted (Mateyko, 1965). In general, *Arbacia* embryos developed in papain solutions but the effects were more drastic than in trypsin, and for survival a thorough washing was needed. Discrepancies in the literature as to the efficacy of trypsin in lysing the fertilization membrane or effecting cell dispersal thus may be partly explained by the different dose levels, purity, time, and duration of exposure to the enzyme.

Berg (1958) achieved dissolution of the fertilization membrane of *Dendraster* by 0.1% protease (non-specified) but needed 5% to get disaggregated blastomeres. In 1959 Nomoto and Narahashi purified a protease elaborated by *Streptomyces* *griseus* which they called Streptomyces protease G. According to Hiramatsu and Ouchi (1963) this enzyme, now called Pronase P, had four active proteolytic fractions. Mintz (1962) reported that 0.5% pronase could be used to safely remove the zona pellucida of mammalian egg cells and Gwatkin and Thomson (1964) used it to disperse both embryonic and adult cells of mice. Later it was shown that pronase was a desirable cell-dissociating enzyme for frog renal tissues (Mateyko and Kopac, 1965).

The effects of pronase on sea urchin development were of interest. Development proceeded normally through the pluteus in 2.5 mg./ml. solutions but when the fertilization membrane lysed, as it did frequently, separation of blastomeres occurred. When eggs were immersed in pronase (0.25 mg./ml.) prior to fertilization, a reduction in cortical consistency was noted and cytolysis followed. Cortical changes elicited by trypsin pretreatment are described by Runnström (1948). Thus the fertilized egg is resistant to the proteolytic action of pronase. Sperm cells, on the other hand, remained motile for several hours and capable of fertilization at the same concentration that caused extensive damage to ova. This variety of response, however, is not unusual when the literature is examined. To reiterate briefly, Runnström and Krizat's (1962) data demonstrated that trypsin elicited cytoplasmic changes in consistency which affected the adherence or elevation of the fertilization membrane. This may also be true for pronase. In addition, when lysis of the membrane occurs, separation of blastomeres is not an unforeseen event. When it is intact, normal plutei develop in pronase solutions. Moore's (1949) data indicate that the time and dose factors for proteolytic action are of importance in that trypsin (and most likely pronase, too) may eventually digest the hyaline layer, thus facilitating separation of cells.

The fact that sperm were so little affected by pronase suggests that pronase affects a cytoplasmic target. Of all the enzymes studied, pronase is the only one in which normal growth and survival continued for two days without washing.

Although sodium taurocholate is not an enzyme, it is a surface-acting agent. Moreover, Rojas (1965) states that the "cell surface protein integrity" generally impaired by proteolytic enzymes is essential for the maintenance of the properties and physiology of the cell membrane. Accordingly, it is considered along with the proteases. Sodium taurocholate, because of its well-known action on lipids of cell membranes, usually brings about lysis of cells. It causes gross changes in the surface structure of erythrocytes (Ponder, 1961) and may do this by enhancing lipolytic action by uncoupling lipase diglyceride complexes (Fritz and Melius, 1963). It may be, in general, an activator of lipases.

There is only one paper, that of Genther and Schmidt (1931) on the effect of this substance on echinoderms. They conjectured that there might be a growth stimulant effect on *Arbacia*. With a mixture of taurocholate and glycocholate they noted that the rate of division and retardation of development were proportional to the concentration of the bile salts.

Our results show that in low concentrations sodium taurocholate (0.2 mg./ml.) did not enhance or retard embryogenesis, while in stronger solutions there was a cleavage arrest and death at the blastula stage with pronounced cellular swelling and erosion of the surface of cells. Similar surface denaturation of frog renal tumor cells occurred after exposure to sodium taurocholate. Even concentrations as low as 0.0003 *M* brought about cytoplasmic alterations (Mateyko and Kopac, 1965).

Allied studies bear mention. Gustafson and Sävhagen (1949) noted that anionic detergents by virtue of their surfactant properties induced radialization in sea urchins. Hagström and Holman (1965) concluded from their work on the effect of free fatty acids on the fertilization of sea urchin eggs that the promoting or negative effects are structure-dependent. The retarding effects of oxidized lipoic acid, especially after blastulation and at gastrulation of sea urchins, are described by Wolfson and Fry (1965).

DNA

The more recent accounts of DNA levels in the echinoid eggs are those of Hoff-Jørgensen (1954), Agrell (1958b), Nigon *et al.* (1963), Troll *et al.* (1964), and Pikó and Tyler (1965), which concur that the cytoplasm contains sizably more DNA than the nucleus, and that the deoxyriboside material is transformed into nuclear DNA during cleavage. In their review Monroy and Maggio (1964) summarize the wealth of biochemical evidence which indicates that the synthesis of DNA in the sea urchin embryo begins quite early after fertilization and may draw upon the precursors resident in the cytoplasm of the mature egg.

Although there is no conclusive evidence whether the intact macromolecule of DNA or one of its degradation products enters a cell which is exposed to a medium containing exogenous DNA, there is no doubt that it does exert a cellular effect. Gartler (1959, 1960), using labeled DNA and cells in tissue culture, showed that a small amount of intact DNA was taken up while some was degraded. The uptake of large fragments of DNA under *in vitro* conditions was confirmed by Chorazy *et al.* (1960), Bensch and King (1961) and Schimizu *et al.* (1962) using Ehrlich ascites, strain L, and Ehrlich ascites cells, respectively. To illustrate further, the penetrability of DNA (2 to 100 mg./ml.) into HeLa cells was demonstrated by Borenfreund and Bendich (1961). H^3DNA, DN-ase-labile, localized in nuclei. Gosse *et al.* (1965), however, do point out that tissue DN-ases degrade exogenous DNA which is injected into mammals. Ledoux (1965) reviews the area of the uptake and subsequent metabolism of DNA by living cells.

By far one of the most stimulating papers is that of Mazia (1949). He noted that the occurrence of cytoplasmic DNA and DN-ase was far above the amount borne by the sperm cell and suggested that the excess DNA was non-genetic. Using *Asterias forbesii* as the developing form, Mazia observed that embryos were stopped at the gastrula stage with homologous DNA (sperm) but not from a heterologous sperm source (*viz. Arbacia*). Thus development was normal in foreign DNA. Hörstadius *et al.* (1954) expanded this facet of work. They extracted DNA from several echinoids and found a variety of responses. DNA from the same species, *Paracentrotus lividus*, did not arrest development but that from *Echinocardium cordatum* was more injurious, etc.

Eggs of *Paracentrotus* developed in DNA solution but many died; in 0.25%, the pluteus stage was reached; in 0.5% the gastrula-prism stage was reached. Moreover, animal halves were animalized if raised in DNA solution. Microinjection of DNA solutions into the egg cytoplasm was also performed. If the droplet did not mix with the cytoplasm, the eggs were fertilizable, development proceeded normally, and the droplet was then extruded.

If the droplet diffused through the cytoplasm, an activation occurred which was followed by cytolysis.

Exogenous DNA has been added to sea urchin embryos as a means of counteracting the developmental arrest produced by actinomycin D (Lallier, 1963a, 1963b). The interaction is discussed in the section on the actinomycin effect. DNA, when added to *Arbacia* embryos at concentrations below 0.05 mg./ml., did not alter appreciably the rate of development or morphological patterns, but at double this level, the growth retardation was evident. In our experiments, *Arbacia* embryos were sluggish at the prism stage with some deaths at the pluteus at 0.1 mg./ml.

Although skeletal differentiation occurred, often the gastrular stage was maintained. Thus the mesodermal derivatives are not inhibited although there is some disruption in morphogenesis. This may be possibly a facet of "animalization" according to the descriptions of this phenomenon by Hörstadius (1949).

DN-ase

The direct *in vivo* or *in vitro* effect of exogenous DN-ase has not been as popular a study as that of RN-ase. A comparison of available data is not satisfactory because of the variety of responses elicited. The effect of DN-ase on cellular DNA of living cells was reported by Kaufmann and Das (1955) who found no growth interference with the addition of DN-ase to root tips, and suggested that the molecule was probably unable to penetrate. The injection of DN-ase did not alter the Feulgen reaction or basophilia in amoeba (Brachet, 1959). The addition of acid DN-ase to embryonic chick cells in culture elicited some mitotic inhibition and a mitochondrial modification (Chèvremont and Chèvremont, 1957). De Lamirande (1961) observed a disappearance of DNA from tumor cell nuclei upon injection of DN-ase to mice with ascites. The incorporation of H^3-thymidine and oxyuridine was depressed only slightly by the addition of DN-ase to HeLa cells in culture (Feinendegen *et al.*, 1961).

In 1948 Mazia *et al.* demonstrated that most of the DN-ase of the unfertilized *A. punctulata* eggs was cytoplasmic. He also reported that cytoplasmic DNA and DN-ase were far above that borne by the sperm cell (Mazia, 1949). Moreover, nuclei isolated from cleavage stages had high levels of DNA polymerase (Mazia and Hinegardner, 1963).

Since our *Arbacia* raised in sea water containing DN-ase showed a retardation of growth and activity, it is clear that the commercially available macromolecule is effective. The effect of the exogenous DN-ase appears to be a non-specific one on *A. punctulata,* in that at high levels animals die while in lower levels (0.05 mg./ml.) only the activity but not morphogenesis is curtailed. A block in embryogenesis by the exhaustion of DNA, as suggested by Moore (1955, 1958), which occurs to a certain extent at the blastula, could possibly be accelerated by DN-ase.

RNA

Since it was conjectured that the inhibiting role of cobalt might be reversed by RNA, the effects of both RNA and RN-ase were determined. One can only emphasize the complexities of RNA metabolism in the development of the sea urchin since a full discussion is beyond the scope of this paper (*vide:* Brachet *et al.,* 1963; Comb and Brown, 1964; Gross and Cousineau, 1964; Gross *et al.,* 1964, 1965; Hultin, 1964; Brachet, 1965a; Comb *et al.,* 1965; and Whiteley *et al.,* 1966).

The relatively large stores of RNA that are present in the fertilized egg (Immers, 1960; Pasteels *et al.,* 1958) and the fact that a limited RNA synthesis occurs from fertilization to blastulation (*vide supra* and review by Monroy and Maggio, 1964) make it unlikely that exogenous RNA, especially in small quantities, can exert any striking effects on developing *Arbacia* zygotes.

In 1957 Niu and Douglas added RNA extracted from *Raia erinacea* to 2-cell embryos of *Styela* and *Arbacia* and found that the rate of development increased with the addition of 5 μg. to 100 μg./ml. for *Styela* and 4 to 16 μg./ml. for *Arbacia.* Our results are in general agreement with these observations, the acceleration being evident at 10 μg./ml. and a definite retardation at 0.1 mg./ml. Developmental aberrations were not produced. The effect of RNA on *Paracentrotus* is reported by Lallier (1964b). The duration of exposure was 30 minutes in 1 mg./ml. RNA is described as inducing radial symmetry and augmenting animalizing effects. We found, however, that the solubility of RNA, unlike RN-ase, was poor at 1 mg./ml. and in order to have an effective solution, a lower concentration had to be used. Very recently Lallier (1966) reported that sRNA (1 mg./ml.) even when subjected to prolonged (15 hours) alkaline hydrolysis exerted a radializing effect on *Paracentrotus lividus* embryos. Cytidylic acid also had this effect but not hydrolyzed thymus RNA.

In a study of the effect of RNA on development of *Strongylocentrotus purpuratus,* Whiteley *et al.* (1965) reported that RNA competes in the binding with DNA, and that RNA from ova and blastulae, and hatching embryos competed less strongly than pluteus RNA. This RNA is mRNA.

In addition, Lansing and Rosenthal's (1952) observation that RNA is present at the cell surface of *Arbacia,* and may participate in ionic transport, is of interest.

It is generally accepted as a dictum that organized nucleoli represent a major repository of nuclear RNA unless mitoses are too rapid, as during embryogenesis, to permit the assembly of RNA into this organelle. In amphibian development Brachet (1964) and Brown and Littna (1964) demonstrated that the evolution of discrete nucleoli at gastrulation coincided with the synthesis of ribosomal RNA.

It is surprising that in the wealth of work on echinoderm development no definitive study has been made on nuclear morphology, specifically on the nucleolus during embryogenesis. In *Paracentrotus miliaris,* Agrell (1958a) pointed out that no nucleoli were noticed up to the 32-cell stage. The chemical study of Cowden and Lehman (1963) on the development of *Lytechinus verigatus* (*sic*) comments on the lack of nucleoli at the fourth cleavage and even in the mesenchyme blastula, and reports that nucleoli appear for the first time at the early gastrula stage. There is, however, more cytoplasmic RNA after the fourth cleavage. Harris (1961) in an electron microscopic study of mitosis (16 to 32 cells) describes in the interphasic blastomere nuclei of *Strongylocentrotus* "a number of dense ovoid bodies of unknown significance." Thus a systematic cytological analysis of nucleoli, documented by photographs, from the 2-cell stage through gastrulation has to my knowledge not been done for any echinoid. One may conjecture, nonetheless, that during embryogenesis RNA synthesis does occur even before visibly organized nucleoli appear.

RN-ase

That ribonuclease can penetrate living cells and elicit a diminished cytoplasmic basophilia is now well-established (Brachet, 1955a, 1955b; Firket *et al.*, 1955). To illustrate, in *Asterias glacialis* oocytes, a diminution in basophilia was noticed after RN-ase (1 mg./ml.). Brachet (1955b) thus concluded that RN-ase penetrated living cells and thus brought about a cessation of mitosis and an inhibition of incorporation of amino acids into proteins.

The literature describing the effect of RN-ase on echinoderm development is relatively sparse, but agrees in general that cleavage arrest is a principal phenomenon. Leone's (1960) studies on *Arbacia punctulata* development in RN-ase (crystalline, 1%) indicated that the most sensitive stage was the fertilized egg, only 20% of the embryos reaching the pluteus. In our experience this was a toxic dose. Ledoux and Metz (1960) also noted an inhibition of sea urchin cleavage by RN-ase but their concentrations were less. The degree of inhibition depended not only on the dose but on the commercial source of enzyme. In *Lytechinus variegatus* but not especially *Arbacia punctulata,* there was a "collapse and partial lysis" of the fertilization membrane. RN-ase at 1.5×10^{-4} *M* definitely exerted a cleavage block.

In our experiments on *Arbacia,* below 0.1 mg./ml. RN-ase, development appeared to run parallel to normal; between 0.1 to 0.12 mg./ml. a slight developmental retardation was produced but normally differentiated plutei resulted. Thus no striking morphogenetic changes ensued when non-toxic doses were applied.

The possible surface effects on RN-ase should be considered. Lansing and Rosenthal (1952) described RNA at the cell surface of *Arbacia.* RN-ase could attack this region.

Cormack (1966) demonstrated that RN-ase (1.5×10^{-3} *M*) conjugated with fluorescein isothyocyanate 3 minutes after fertilization, blocked the cleavage of 90% of *L. variegatus* and *A. punctulata* while the rest were arrested at 2- or 4-cell stage. This is, however, a high dose. The fluorescein-labeled RN-ase was absorbed to the fertilization membrane and hyaline layer, but was not detectable within the egg. However, during development from the blastula to the pluteus, the labeled RN-ase was incorporated into cytoplasmic droplets by cells of the digestive tract. The reversibility by washing also pointed to a possible surface effect of RN-ase.

Interpreting these data, it is clear that small amounts of exogenous RN-ase do not alter appreciatively the development of the echinoid zygote. Larger, but non-toxic, amounts slow down the embryogenesis but do not shut down mitosis or bring about any changes in the direction of morphogenesis. Larger amounts of RN-ase, which are cytotoxic, may effect this *via* a massive degradation of all types of cellular RNA and thus a disorganization of RNA or even protein metabolism. A surface effect on ionic transport is also to be considered.

Thymine

One may conjecture that thymine may function as a growth factor by virtue of its conversion to 5-hydroxy-methyluracil or to thymidine (Abbott *et al.*, 1964), and subsequent utilization as a DNA precursor, but experimental evidence shows that it is generally inhibitory. It slows the rate of cell division in onion root tips, (Deysson, 1954), and may inhibit the timing of mitosis as adenine does (Biesele *et al.,* 1952; Brachet, 1959).

According to Brachet (1959) purines and pyrimidines inhibit growth and regeneration in *Acetabularia,* the pyrimidines being less inhibitory. In general, sea urchin embryos can utilize nucleosides in biosynthetic reactions (Nemer, 1962). The morphogenetic effects of some nucleotide metabolites on sea urchin development have been assayed by Markman (1964). Generally, in low concentrations a slight acceleration of development may occur. But cleavage retardation by pyrimidine derivatives has been reported by Stearns *et al.* (1962) for *Strongylocentrotus.*

In sea urchin development, low concentrations of thymine are mildly retarding to growth, but development proceeds normally. The higher concentrations appear to be particularly inhibitory at the transition from blastula to gastrula. Although the cleavage block is striking in thymine solutions (0.1 to 0.4 mg./ml.) and not alleviated by RN-ase or DN-ase, the binding of thymine must be weak since recovery is rapid upon removal of embryos to fresh sea water. The very striking retardation of differentiation and growth elicited by DNA plus thymine may be explained perhaps by the toxicity attributed to pyrimidine bases (Biesele *et al.,* 1952) and nucleic acids (Smith, 1964). It should be mentioned, however, that the block to the development of *Paracentrotus* at 16 to 32 cells by 5-fluorouracil was reversed by thymine and thymidine. According to Lallier (1965) this suggests that the pyrimidine analogue interferes with the elaboration of thymine necessary for the synthesis of DNA.

Cobalt

In 1923 Hoadley made a study of the effect of metal salts, including cobalt chloride, on the fertilization reaction in *Arbacia,* and reported a cleavage toxicity for cobalt in 1:100 concentration. In the present experiments, the retarding effects on cleavage are reversible if the concentrations are low (0.01 mg./ml.) and the treatment does not extend beyond 5 hours. At higher concentrations (to 0.02 mg./ml.) the drastic exogastrulation is irreversible. Rulon's (1956) study on sand dollar embryos revealed that cobalt brought about developmental modifications at low concentrations and inhibited cleavage and elicited exogastrulation at higher concentrations. More recent work confirms these inhibitions of development by cobalt (Mateyko, 1961). Our data have shown that cleavage, *per se,* is not blocked by cobalt in low concentration, but that embryogenesis proceeds at a slower rate.

Since the cobalt effect is produced with equal facility by cobalt chloride and cobalt glycine, the effectiveness of the cobalt ion, rather than the glycine moiety, is clear. According to Mastrangelo (1966) glycine does not have any effect on the development of various echinoids.

Rulon (1956) had noted that there was a lack of cohesiveness in the cobalt-treated *Dendraster* embryos. A tendency toward cellular disaggregation was also evident in *Arbacia* larvae, but the phenomenon was never very striking. Our results show that at gastrulation, the morphogenetic directions are misrouted, and irreversibly so. It thus appears that the unique cobalt effect is locked in at gastrulation when a dramatic change in form, "the pollen grain" exogastrulae are produced. This alteration is so striking and so characteristic that it may be described as the specific cobalt effect. Among the other structural effects were: cleavage retardation and irregular deposition of cells, polar elongation, exogastrulation, inhibition of proper differentiation of spicules, and swelling ("bloating"). These aberrations are similar not only to those reported by Rulon (1956) for *Dendraster*, but also closely resemble the original lithium effect of Herbst (1892). Likewise, the cobalt-induced exogastrulae are closely identifiable with the Hörstadius (1949) figures on vegetalization brought about by lithium. In some instances, *Arbacia* embryos showed a slight degree of radialization, the phenomenon being characteristic of weak animalizing agents.

It has been known since the publication of Lindahl's (1936) monumental work, that glycolysis and respiration are inhibited in echinoids by lithium. The paper, although 30 years old, bears careful re-reading. In Lallier's (1964a) survey on the lithium effects in echinoderms, it is clear that many aspects of cell metabolism are affected by lithium—respiration, glycolysis, ATP synthesis, nucleoside metabolism, etc. Comparable work on cobalt is sparse.

The metabolic target of cobalt is difficult to pinpoint because of the many intracellular effects. To specify, Laskowski (1961) points out that DN-ase I requires cobaltous ions for activity whereas soybean DN-ase is inhibited by cobalt. It has long been evident that cobalt is inhibiting to several enzymes. Levy *et al.* (1950) demonstrated the inhibition by cobalt of succinoxidase, choline oxidase, cytochrome oxidase, catalase, and choline dehydrogenase and succinic dehydrogenase in mammalian tissues. Cobalt also acts as an inhibitor of purified α-oxoglutarate dehydrogenase *in vitro* (Webb, 1964).

A generalized biological action of cobalt was interpreted by Webb (1962) to be an inactivation of systems that are dependent upon the reversible interconversion of lipoic acid and dihydrolipoic acid. Rulon (1963) suggested that cobalt ions became attached to SH groups at the surface of *Strongylocentrotus* eggs. In their studies of cellular morphogenesis in *Psammechinus*, Gustafson and Wolpert (1962) account for structural changes in general by a "change in contact between individual cells and between cells and the hyaline membrane." Whether exogastrulation is attributable to cobalt-induced metabolic disturbances directly, or is due to a secondary change in the surface properties of cells is unclear. Weiss (1950) has stressed the importance of the latter in differentiating systems where exogastrulation occurs.

Lithium affects ribonuclease (Bigelow and Geschwind, 1961); cobalt inhibits bacterial ribonuclease (Muira and Nakamura, 1951). It is not evident, however, whether the inhibiting effect of lithium bromide on ribonuclease can in any way be compared to the inhibiting effects of cobalt chloride on ribonuclease.

The observations that cobalt inhibited bacterial RN-ase (Muira and Nakamura, 1951), that it produced persistent nucleoli in tissue culture (Heath, 1954), that it inhibited intracellular RN-ase in *Tetrahymena* as well as crystalline RN-ase (Roth, 1956), that it brought about formation of extra micronucleoli in *Vicia faba* (Komczynski *et al.*, 1963) all suggested that cobalt was involved in the metabolism of ribonucleic acid. In our experiments, the addition of RN-ase, which in itself is growth-retarding in concentrations greater than 0.05 mg./ml., produced more active, but still malformed exogastrulae. The ameliorating effects of RNA were also quite slight, although Lallier (1946b) found that lithium-vegetalized sea urchins were "equalized" by the addition of RNA. He indicates that soluble RNA influences morphogenesis in *Paracentrotus* by regulating protein synthesis. Possibly when net nuclear RNA synthesis is established at gastrulation and major morphogenetic events occur (Brachet, 1965a, 1965b) the interference of the cobalt ions in cell metabolism seems evident. Moreover, the addition of DNA and DN-ase did not reverse the effect. In fact, the addition of DNA to cobalt-treated embryos arrested growth of *Arbacia* even earlier.

One can speculate, then, if nuclei of the sea urchin are irreversibly differentiated at gastrulation, the cobalt effect may be stabilized in these differentiated nuclei.

Actinomycin D

Muira (1953) found that actinomycin A at 100 mg./ml. slightly retarded the rate of cleavage in *Arbacia punctulata*. Later Wolsky and Wolsky (1961) observed if fertilization occurred in actinomycin (25 μg./ml.), there was a retardation, anomalous growth, and death before gastrulation. Our data showed that 5 μg./ml. actinomycin D was effective in arresting most embryos at the blastula (Mateyko, 1965). A series of more detailed papers on echinoid morphogenesis followed which generally concurred that actinomycin D caused a dose-dependent inhibition of larval cleavage and differentiation (Brachet *et al.*, 1963; Lallier, 1963a, 1963b; Markman and Runnström, 1963; Gross and Cousineau, 1964; Markman, 1964; Guidice and Hörstadius, 1965). The present morphological results, while agreeing essentially, do show some departures that bear discussion. These involve the effective dose level for retardation, the specific morphological effects and the stage of blocking, and attempts at amelioration by other molecules.

The sample of actinomycin D (provided by Merck, Sharpe, and Dohme) has a low aqueous solubility, especially in sea water. Because of this property, generally solvents such as acetone, ethanol, or propylene glycol, etc., have been used for the initial preparation of stock solutions. Although Gross and Cousineau (1964) made a stock solution of 246 μg./ml. in sea water, our sample on standing showed particles in suspension at 0.15 mg./ml. Thus our stock solutions were made at lower concentrations in distilled water (100 μg./ml.) and in 95% ethanol (0.5 mg./ml.) to insure non-precipitating solutions.

In our experiments the developing *Arbacia* embryos were susceptible to actinomycin D at quite low levels (2 to 3 μg./ml.) and it is our thesis that the precipitability of actinomycin D in sea water may be a factor. That actinomycin D is biochemically active at concentrations much lower than those that bring about developmental aberrations is presented by Gross and Cousineau (1964) who indicated that a concentration as low as 1.4 μg./ml. was effective in inhibiting the uptake of C^{14}-uracil by 64% (the full effect of 94% inhibition occurring at 24 μg./ml.). Our studies on the actinomycin effect were designed, therefore, to check the "marginal" concentration for response such as has been described by Reich *et al.* (1962) for HeLa cells.

The specific morphological effects and stage of blocking again are of interest in that lower concentrations of the substance than previously reported have been effective, and development has been followed for several days or until the embryos succumbed.

At 2 μg./ml., a marginal dose, there was an inhibition in that *Arbacia* embryos were slower cleaving and thus morphologically younger than the controls, and while they did reach a pluteus stage, the growth of the arms was retarded. At levels below 3 μg./ml. cleavage was retarded but proceeded through gastrulation, and some abnormalities occurred during this transition. The striking effect was noted at 4 μg./ml. Up to the point of gastrulation, cleavage proceeded slowly, but gastrulation *per se* was abnormal and further differentiation to the pluteus stage was blocked in that skeletal and arm formation was inhibited. Lallier's (1963a, 1963b) data on *Paracentrotus lividus* grown in an actinomycin concentration of 5 μg./ml. are in agreement in that asymmetric gastrulae were produced without spicules. These recovered when placed into fresh sea water.

Gross and Cousineau (1964) reported that between 6 to 20 μg./ml. actinomycin D, cleavages were faster and at 6 μg./ml. were at the same rate as the controls, but even at this level irregular blastulae were produced. Some failures of gastrulation occurred even at 1.4 μg./ml. Our experiments demonstrated that even at 3 μg./ml., the retardation in early cleavage was indisputable and no enhancement of division was found even at the 2 to 4 μg./ml. level. Disorganization in the regularity of cleavage appeared quite early even at 4 μg./ml. in that there was an irregular disposition of surface cells even before the morula stage so that a "lumpy" surface was characteristic.

The effects of low levels of actinomycin D, however, are even more apparent when embryos are maintained beyond 24 hours. We found that while the early cleavage stages showed retardation, the older organisms were even more striking in that bloated embryos survived for 5 days, spinning about but never producing arms, skeleton, or other evidence of further differentiation.

In the present experiments with 20 to 30 μg./ml. of actinomycin D, our *Arbacia* embryos died at the morula stage, while at 40 μg./ml. they did not reach the sixth cleavage. Even at 8 μg./ml., the attempt at gastrulation often failed. The fact that Gross and Cousineau (1964) raised embryos, albeit with some abnormalities, in concentrations of 65 μg. to 120 μg./ml. may be explained by a difference in the solubility of the samples.

We are in agreement with Lallier (1963b) in that a dose-dependent inhibition of development is present and that a fresh sea water milieu enhances survival if the concentration of the antibiotic does not exceed 10 μg./ml. But even at this level, abnormalities of gastrulation occurred. Gross and Cousineau (1964) report that at 65 μg./ml. the embryos are inert multicellular masses compared to controls which are gastrulae. Our results indicate that at 20 μg./ml. development could not proceed beyond the morula stage. In order to get the morphogenetic block at the blastula, concentrations no greater than 8 μg./ml. had to be used. Under these conditions, survival with irregular and very slow cleavages proceeded up to 80 hours.

Olsson (1965) noted that actinomycin C disturbed the "ordered mesenchyme" when administered at the blastula stage (256–512 cells) in *Echinus esculentus.*

One of the most striking evidences of differentiation following the onset of gastrulation is the formation of spicules. According to Okazaki (1965) the primary mesenchyme cells contribute to the elaboration of a spicular matrix within which a calcareous granule is elaborated. With actinomycin blocking at the blastula, the mesenchyme fails to form (Gross *et al.*, 1964) and thus the lack of a skeleton in the actinomycin D environment is explained. In *P. lividus* Lallier (1963a) observed that while primary mesenchyme cells did develop in blastulae (actinomycin = 10 μg./ml.), no progress beyond the morula and no spicules occurred.

If the embryos were washed, survival was enhanced, but migration of the primary mesenchyme into the blastocoele was delayed (Markman, 1964). Thus the actinomycin block to skeletal formation may lie in the inhibition of the elaboration of a spicular matrix by the primary mesenchyme cells. The defective arm formation is readily explained by Hörstadius' (1949) observation that the arm has to be predetermined in the ectoderm before the spicule reaches the ectoderm and the skeletal rod is needed to exact an influence to form an arm.

Although the level of actinomycin D (40 μg./ml.) used by Guidice and Hörstadius (1965) on *P. lividus* was higher than our levels (8 μg./ml.) the sequelae (blastular arrest, defective arm formation, and inhibition of skeletal formation) were similar.

Lallier (1963a, 1963b) and Markman and Runnström (1963) point out that actinomycin (like lithium) enhances the vegetalizing properties of the embryo. Runnström and Markman (1966) also cite that exposure to actinomycin D (12.5 μg./ml.) at the 8-cell stage for 5 hours causes animalization which is a "secondary effect of retarded gastrulation," and that lithium (0.033 *M*) to some extent counteracts this.

By now an enormous literature has grown on the biochemistry of the actinomycin D effect and no attempt will be made to survey the literature systematically. The bulk of work leads to the conclusion that the effect of actinomycin is due to the irreversible complexing with guanine-containing binding sites in DNA (Goldberg *et al.*, 1962; Kahan *et al.*, 1963). Thus the mechanism of inhibition of mRNA synthesis is brought about by the preferential binding of actinomycin to the primer DNA excluding a competition with precursors or a direct effect on RNA polymerase (Cavalieri and Nemchin, 1964). The biochemistry of actinomycin, and the complexing of it with DNA and purines, and the correlation of the structure and function of its complexes are beautifully demonstrated by Reich (1963, 1964).

Observations of the actinomycin D effect on echinoids on a molecular level abound since this compound has yielded a rich harvest of information on sequential biochemical events in development. To illustrate briefly, Gross and Cousineau (1963, 1964) noted in *Arbacia punctulata* that actinomycin treatment inhibited RNA turnover, but that the incorporation of amino acids into protein was unaltered and DNA synthesis continued although at a slower rate. In *Paracentrotus lividus,* Brachet (1963) and Brachet *et al.* (1963) observed that while actinomycin D slightly delayed cleavage, it did not inhibit the incorporation of C^{14}-leucine into proteins. Recently, Billiar *et al.* (1966) pointed out that actinomycin D inhibited protein synthesis in *A. punctulata* noticeably only after the early blastula stage.

In our studies the only agent in reasonably physiologic levels which appeared to ameliorate slightly the actinomycin D effect was DNA (0.2 mg./ml.). Thymine

(0.05 mg./ml.) merely enhanced survival while the effect of exogenous RNA was inconclusive. In experiments on *P. lividus,* Lallier (1963a, 1963b) demonstrated that 0.5 mg./ml. of DNA was protective against 10 μg./ml. of actinomycin D, but was ineffective if higher doses of actinomycin were used.

Kersten *et al.* (1960) indicated that purines and pyrimidines could displace actinomycin from the site in the living cell, and as a rule, the addition of DNA can ameliorate the actinomycin D effect, the actinomycin reacting directly with the DNA. According to Hurwitz *et al.* (1962) the inhibition of RNA polymerase by the antibiotic may be reversed by increasing the concentration of DNA. Yeast RNA at 100 times the concentration of DNA is only partly effective in relieving the inhibition (Goldberg and Rabinowitz, 1962). In ascites cells some of the inhibiting effects of actinomycin D (0.1 μg./ml.) are ameliorated by the addition of 200 μg./ml. DNA (Niu, 1963).

An interpretation of the enhancement of survival in actinomycin D by thymine cannot be provided at present. Setlow *et al.* (1963) demonstrated that one thymine dimer per 350 μ strand of DNA acted as a block to further DNA synthesis. In *E. coli* the block was permanent, while in other cells, it was only temporary. Kersten *et al.* (1960) indicate that thymine, as well as RNA could somewhat ameliorate the actinomycin block in *Neurospora crassa.*

Wheeler and Bennett (1962) suggested that actinomycin was preferentially fixed by the DNA of nucleoli. And in 1963 Reynolds *et al.* (1963a) reported a unique nucleolar effect of actinomycin D on Chang liver cells in culture, that of a separation of the pars amorpha and nucleolonema into "nucleolar caps."

Since the presence or absence of organized nucleoli in early echinoderm cleavage has not been fully explored, the pin-pointing of a cellular organelle as the target of actinomycin D, other than to a DNA-containing structure, is premature.

It is suggested that where these low concentrations of actinomycin D are eliciting a morphological response that there is a limited and differential binding of DNA by the antibiotic, and thus a differential synthesis of mRNA. Since skeletal formation and the formation of arms in the plutei are inhibited, it seems likely that the binding of DNA sites by actinomycin D is initially at very specific sites.

4-nitroquinoline N-oxide

The effects of 4-NQ on cell metabolism are many. According to Fukuoka *et al.* (1959) and Ono *et al.* (1959) in mouse ascites cells there was an inactivation of —SH enzymes, a subsequent inhibition of glycolysis, and as a result, a reduction in adenosinetriphosphatase, and thus no regeneration of ATP. Accordingly, the uptake and incorporation of amino acids into protein and the uptake of P^{32} into nucleic acids were depressed. Simply stated, the consequence of decreased DPN and ATP levels was an inhibition of protein and nucleic acid synthesis.

Endo *et al.* (1959) reported that 4-NQ produced intranuclear inclusion bodies in Chang liver and mouse kidney cells. Moreover, the cytological effects of 4-NQ (10^{-5} *M*) (Reynolds *et al.,* 1963a) in that in both instances there was a decrease in nucleolar size, a fusion of nucleoli, and separation of the pars amorpha and nucleolonema to form nucleolar caps (Reynolds and Montgomery, 1964; Reynolds *et al.,* 1964).

It was also proposed by Reynolds *et al.* (1964) that 4-nitroquinoline N-oxide might have the same specific effects on the DNA-dependent RNA production as actinomycin D. Our work suggests that the powerful mitotic arrest produced by 4-NQ on *Arbacia* embryos should be investigated by determining the incorporation of nucleic acid and protein precursors.

Since 4-NQ acted as a strong inhibitor of gastrulation (block at the blastula) at 5 μg./ml., its arresting qualities appear similar to those of actinomycin D. On the other hand, the viability of sperm at quite high concentrations of 4-NQ (0.125 mg./ml.) is of considerable interest. Obviously, the metabolic event which is blocked in the cleaving zygote is inoperable or most likely lacking in the sperm cell. Troll (personal communication) comments that methods which give evidence of RNA polymerase in *Arbacia* eggs do not reveal the presence of this enzyme in sperm cells. This negativity may, of course, be due to technical difficulties. Since there is a paucity of information about sperm nuclear enzymes, speculation about the lack of effect by 4-NQ is hampered. Reviews on general biochemical events (Monroy and Maggio, 1964), actinomycin D and RNA polymerase interaction (Reich and Goldberg, 1964), and the role of nucleic acids and sulfhydryls in echinoid morphogenesis (Brachet, 1964) are helpful only in elucidating these phenomena in eggs or cleaving zygotes.

Thus 4-nitroquinoline N-oxide may prove to be as effective a tool to probe the molecular aspects of sea urchin development as actinomycin D. Its ineffectiveness on sperm viability and inhibiting effect on zygote development are quite striking.

Coumarin

Coumarin is one of the naturally occurring active agents that is found in seeds and spores and takes its place among the native mitotic poisons such as the alkaloids and essential oils. According to Goodwin and Tanes (1950) coumarin should be thought of as an inhibitor rather than a toxic substance. The review by Soine (1964) lists some 31 different physiological activities of coumarins and indicates that many more may be forthcoming. In its natural state coumarin functions as a plant growth regulator, possibly at the level of the cytochromes.

Coumarin has long been recognized as an inhibitor of seed germination (Sigmund, 1914). Recent work attributes a growth-stimulating quality to coumarin (Knypl, 1964a, 1964b). Knypl (1964b) suggests that coumarin acts either through changes in the mechanical properties of the cell wall or through the metabolism of cells. He also indicated the possibility of a coordination of mRNA synthesis by coumarin (Knypl, 1965).

Since coumarin is a plant product its use on animal tissues has not been extensive aside from the studies on warfarin, a coumarin derivative. In rats, coumarin compounds generally antagonize vitamin K, uncouple oxidative phosphorylation, and thus affect the energy supply of the cell (Martius, 1961). By uncoupling oxidative phosphorylation, protein synthesis may be inhibited. One of the more recent accounts by Pool and Borchgrevink (1964) reports on warfarin as inhibiting the incorporation of amino acids into protein of the liver.

In the present experiments on sea urchin embryos, the mitotic inhibition by coumarin was demonstrated in the paucity of cells in the 40-hour-old embryos. In addition, coumarin specifically and irreversibly interfered with the proper gastrulation and differentiation.

Cornman (1947) pointed out that coumarin suppressed the spindle, effected a splitting and shortening of chromosomes, and also prevented the inception of mitosis.

Because its full range of physiological activities may be so widespread (cytogenic, cytological, estrogenic, etc.; Soine, 1964), it is doubtful that its precise metabolic effect in *Arbacia* can be at present pinpointed.

Mescaline

The area of pharmacological effects of mescaline is a much more intensively studied one than that of the cellular role. Upon administration, oxidation of lactate, pyruvate, glutamate, and glucose is inhibited in guinea pig brain (Neff and Rossi, 1963). Whether injected intravenously (Neff *et al.,* 1964) or given orally (Charalampous *et al.,* 1964) to mammals it is agreed that it is deaminated to a physiologically inactive compound, 3, 4, 5-trimethyoxyphenylactic acid. It also has an inhibiting effect on the histaminolytic power of diamine oxidase (Carlini *et al.,* 1965).

Because of its striking neurological involvements in man, the cytological effects of mescaline have been noted principally in vertebrate tissues. A cytotoxic effect in tissue culture was reported by Painter *et al.* (1949) for a variety of substance that influence the activity of the nervous system. Lettré and his associates noted that 5 μg./ml. inhibited cell division and brought about vacuolization within the plasma membrane of chick fibroblasts (Lettré. 1948; Lettré and Albrecht, 1941, 1943; Lettré *et al.,* 1941). A direct cellular response as chromophobia of neurons, clumping of Nissl in neuroglia, etc., has been reported by Cazzullo (1963). Hoffer and Osmond (1960) state that it becomes bound to proteins of mitochondria and microsomes. It is also bound strongly by nuclei, weakly by microsomes and may be incorporated into tissue proteins (Neff and Rossi, 1963).

In our studies it was clear that in low concentrations of mescaline, development was slowed down. At the pluteus stage there is striking evidence of the inhibition of growth of the arms and the spicules, especially since a minuscule skeleton is formed in a normal-sized larva. The fact that the mescaline effect is not reversible after gastrulation suggests that a permanent binding or inhibition has occurred. The mechanism of action remains unclear but may be due to an inhibition of some step in the oxidative pathway. To illustrate, Zeuthen (1953) summarized the evidence to conclude that oxygen consumption in echinoids increased just about the time that primary mesenchyme cells appeared. Since the primary mesenchyme cells play a role in the formation of the spicular matrix (Okazaki, 1965) and since mescaline inhibits some oxidative step (Neff and Rossi, 1963), the miniaturization of the skeleton may be explained.

Thalidomide

One of the problems in administration of thalidomide is its insolubility and because of this, it is generally administered as a suspension rather than a true solution. Even without attempts at solubilization, at physiologic levels it decomposes spontaneously to give a mixture of compounds (Williams, 1963). Therefore, it was used in the present experiments in a solution that was often a suspension at concentrations about 0.02 mg./ml. to 0.1 mg./ml. Critical periods in *Arbacia* development during which the thalidomide effect could be evident presumably are: the initiation of cleavage, blastulation, gastrulation, and the inception of differentiation. It was found, however, that these stages were not subject to major disruptions, merely delay. The thalidomide effect, in fact, appeared to slow up at a later stage when the plutei began to show elongation of arms. Another specific effect was the persistence of the fertilization membrane in three-day-old plutei. This indicates that the hatching enzyme which is activated at the blastula (Kopac, 1941) is affected or the susceptibility of the fertilization membrane has been altered. According to Hallberg (1964) the hatching enzyme is present but inhibited in the unfertilized egg; therefore, thalidomide appears to prevent the release of this inhibition or may bring about a denaturation of the membrane which renders it undigestible by the hatching enzyme.

The mechanism of thalidomide action in disrupting normal echinoid embryogenesis is not fully elucidated. For protozoan inhibition, Frank *et al.* (1962, 1963) suggest that thalidomide interferes with the synthesis of diphosphopyridine nucleotides or the reduction of the cytochromes, or it may act at the point where adenine dinucleotide (NAD) is synthesized, or it may interfere with the utilization of NAD and vitamin K in cellular oxidations. Boylen *et al.* (1963) introduce evidence that thalidomide interferes with glutamine metabolism.

Miller (1963) puts the activity of thalidomide as an inhibitor of RNA and protein synthesis, while Vasilescu *et al.* (1964) report that thalidomide interferes with the metabolism of nucleic acids and alters their structure. In our experiments, RNA, RN-ase, DNA, and DN-ase enhanced rather than alleviated the retarding effects of thalidomide. Therefore, it is suggested that a strong binding of thalidomide or one of its degradation products to some cellular organelle occurred, or it induced an aberration in some biosynthetic pathway, and as a consequence normal embryogenesis was altered. The most plausible explanation is a block in the metabolism of glutamine.

Conclusions

For the early work on metabolic activities and cleavage of *Arbacia punctulata* referral is made to the review of Krahl (1950). The bibliography assembled by Harvey (1956) is highly relevant, while the more recent aspects of echinoid development are compiled by Monroy and Maggio (1964).

One other aspect of echinoid development needs to be considered in light of the dramatic exogastrulation elicited by cobalt. Runnström (1928) developed the concept that there are two potentialities in the sea urchin egg, one animal, the other vegetal, each occurring at the respective poles. During normal differentiation, these interact in a controlled manner. By altering the amounts of animal or vegetal blastomeres or by disrupting the metabolism of one of the potentialities a zygote can be directed into animalization (hyperdevelopment of ectodermal structures), or into vegetalization (hyperdevelopment of entomesodermal structures). The first striking example of vegetalization was that of Herbst (1892), which was induced by lithium ions. Various alterations in the direction of animalization or vegetalization by ions, metabolites, etc., have been described, and reviews on this topic have been admirably put together by Ranzi (1962) and Lallier (1964a).

Ranzi (1962) advances a scheme for echinoid development based upon protein denaturation. Presumably, at the animal pole at fertilization, certain denatured proteins set into play the process for animalization. The proteolytic enzymes (ficin, trypsin, chymotrypsin) are animalizing (Hörstadius, 1949, 1953; Moore, 1952). Lallier's (1964a) view is that embryogenesis is directly affected by the inhibiting action of animalizing or vegetalizing agents or *via* "the formation of inhibitors of the synthesis of specific proteins."

In analyzing the effects of a substance on embryogenesis one should exclude substances that are ineffective at moderate concentrations and toxic with massive dose. These generally appear to elicit no graded dose-dependent response. No morphological changes occur, merely death and subsequent lysis of the blastomeres —as was found with sodium taurocholate. Toxic substance may be distinguished as those which show little or no effect below a certain level but exert an overall lethal effect at the proper (usually small) dose. They may act on any one, or on several cellular systems, bringing about complete cessation of function. The effect may be physicochemical as denaturation of proteins by alcohols. Enzymes, especially proteolytic ones, may fit into this category, especially if they penetrate.

Metabolites as normal constituents of the cell should be considered in their own category, that of being part of the cell economy. Herein would be included the enzymes, RN-ase and DN-ase and their respective substrates, RNA and DNA. Their effects would be analyzed in terms of the molecular biology of the cell.

Mitotic inhibitors, whether they act on the cytosome or on the nucleus, may bring about mitotic arrest or retard mitosis. A true mitotic retardant would give a dose-dependent inhibition; not so a mitotic arrestor. Thalidomide was a mitotic retarder. In the category of mitotic inhibitor would fall substances which slow down cell division and, in addition, alter the direction of morphogenesis. For example, cobalt may be considered genotropic in the sense that it disorients development and reorients it in another direction, *i.e.* toward exogastulation. A substance such as actinomycin D, however, which retards cleavage in low doses but firmly arrests it in higher levels and halts differentiation, could be genostatic, *i.e.*, the development of spicules and arms is totally inhibited. The compound, 4-nitroquinoline N-oxide, is genostatic; mescaline and coumarin most likely are genostatic as well.

The action of mitotic retarding agents would be reversible by changing the medium. The effect of genostatic and genotropic substances might be reversible, but irreversibility of action would be characteristic of toxic substances, and of genostatic and genotropic substances past the "point of no return."

Referral is made to Goodwin's (1963) analysis of control mechanisms. He states that there are three levels at which metabolites alter the activities of macromolecules by interaction: at the DNA level which would affect either DNA or RNA synthesis, at the messenger RNA level which would affect protein synthesis, and at the protein level which would affect the enzymes or the physical characteristics of proteins.

Dettlaff's (1964) review on embryonic development, analyzed from the point of view of mechanics of cell division, duration of periods of cleavage, periodicity of morphogenetic functions, etc., is also highly relevant.

SUMMARY

1. For clarity, brevity, and accuracy in comparisons, a series of stages in the early development of the sea urchin, *Arbacia punctulata,* is presented. Each major morphological event is identified by a symbol.

2. Concentrations of ethanol and methanol of 1% or less have no visible effects on embryogenesis except for a slight growth retardation in ethanol. At 5% development was arrested at the morula stage, while at levels greater than 5% fixation of the cells occurred at the 2-cell stage.

3. The effects of papain (1 mg./ml.) on fertilized eggs were similar to but harsher than those of trypsin in arresting development and effecting dispersal of blastomeres. The fertilization membrane, however, was more resistant to dissolution than in trypsin. Upon lysis of the fertilization membrane, naked larvae often fell apart to blastomeres.

4. Sperm remained motile for several hours in pronase solutions (0.25 mg./ml.) whereas egg cells showed striking cortical changes followed by cytolysis. The fertilized egg, however, was more resistant and underwent cleavage in solutions 10 times more concentrated. Pronase often lysed the fertilization membrane within two hours post-fertilization; subsequently, separation of blastomeres occurred. When the membrane remained intact, normal plutei developed. It is suggested that pronase is a more suitable cell-dispersing enzyme than trypsin.

5. Sodium taurocholate was ineffective at 0.2 mg./ml. but at 2 mg./ml., by virtue of surface and internal changes (increasing lipolysis), brought about a rapid death to zygotes.

6. Exogenous DNA, rather than enhancing cleavage, causes a growth retardation at 0.1 mg./ml. with some lethal effects. Differentiation of mesodermal structures, however, continues although the pluteus shape is inhibited. The consensus is that nuclear aberrations are produced.

7. In general, the addition of DN-ase elicits a retardation of activity and cleavage in *Arbacia.* A concentration of 0.2 mg./ml. brings about death in 48 hours without any morphogenetic alterations. These effects may be attributed to a depletion of DNA by the enzyme.

8. At 0.1 mg./ml. and greater of RNA, an inhibition of cleavage is characteristic, but no specific malformations develop. Small levels of RNA (10 μg./ml.) accelerate slightly the rate of early cleavage, probably by a contribution to the cytoplasmic pool of RNA from which it is metabolized.

9. When physiological doses of RN-ase (0.1–0.12 mg./ml.) of RN-ase are added to sea water, *Arbacia* embryos exhibit only a slight retardation in rate of cleavage and a sluggishness in movement, and pass successfully through gastrulation. Larger amounts are cytotoxic, probably due to a massive degradation of cellular RNA.

10. Thymine, in concentrations of 0.04 mg./ml. to 0.4 mg./ml., inhibits development, the effects increasing with increasing doses. The block at gastrulation is striking at 0.4 mg./ml. Recovery by washing is rapid.

11. Cobalt chloride and cobalt glycine are lethal to *Arbacia* embryos at the blastula (0.2 mg./ml.) and at the morula (1 mg./ml.). In below-lethal levels (0.01 to 0.02 mg./ml.), the vegetalization-radialization is striking. Cleavage planes are disrupted, resulting in rough-surfaced embryos. The drastic aberration,

however, becomes evident at gastrulation when bloated embryos develop as exogastrulae. The skeleton is miniaturized. RNA and RN-ase very slightly ameliorate the effect, but RNA and DNA and DN-ase do not. Washing within five hours of immersion prevents exogastrulation.

12. The low solubility of actinomycin D in sea water and its slight precipitation upon standing must be considered in assaying for its dose-dependent retardation of cleavage and differentiation in sea-urchins.

13. Accordingly, 4 μg./ml. of actinomycin D effectively proves to inhibit full development beyond the blastula. The sequelae, blastular arrest, inhibition of spicule and arm formation, are well defined at 8 μg./ml. but this constitutes a toxic overdose. The first morphological evidence, aside from retardation of cleavage, was a disruption in the alignment of blastomeres, even before the morula stage. Reversal of the effect by washing, DN-ase, RNA or RN-ase was not obtained, but some amelioration was provided by DNA or thymine. Because of these events the binding of actinomycin probably occurs at specific sites.

14. Similar to actinomycin D, at 5 μg./ml. 4-nitroquinoline N-oxide, acted as a powerful inhibitor of gastrulation. Sperm cells, however, were viable for several hours at 0.0125 mg./ml. It is suggested that the metabolic block is inoperable in the sperm. The mechanism, inhibition of —SH groups or lack of RNA polymerase in sperm cells, needs to be investigated.

15. Coumarin (*ca.* 0.5 mg./ml.) can effectively act as a block to gastrulation. Although development is highly abnormal, the overall activity, swimming in eccentric orbits, is not curtailed. Which of the multiple physiological effects of coumarin is operable cannot be stated.

16. Mescaline at 1 mg./ml. is lethal to embryos at the gastrula stage. The most striking effect of mescaline (0.1 mg./ml.), aside from its growth-retarding one, is the production of a miniaturized skeleton in a normal-sized pluteus larva.

17. In non-lethal concentrations (0.02 to 1 mg./ml.) thalidomide permits normal although slower morphogenesis through the pluteus. The retardation, however, is not reversible by washing or the addition of RNA, RN-ase, DNA, DN-ase, etc. In many instances the fertilization membrane persisted about the larvae.

18. In evaluating the overall developmental effect of compounds on embryogenesis, substances may be identified, with some degree of overlapping, as: normal metabolites, animalizing, vegetalizing, or radializing agents, as mitotic arrestors or inhibitors, genotropic or genostatic or toxic.

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RADIOACTIVE LABELING OF RNAs OF SEA URCHIN EGGS DURING OOGENESIS[1]

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In studies with sea urchins it is often of considerable value for the investigator to have available unfertilized eggs in which one or more specific substances have been radioactively labeled. The low metabolic activity of the unfertilized egg and its relative impermeability to many substances of biological interest hamper attempts at direct labeling of the shed eggs. For instance, uptake of phosphate (Whiteley, 1949; Litchfield and Whiteley, 1959; Chambers and Whiteley, 1966; Whiteley and Chambers, 1966) and nucleosides (Piatigorsky and Whiteley, 1965; Mitchison and Cummins, 1966; Siekevitz, Maggio and Catalano, 1966) is very greatly suppressed and that of amino acids (Mitchison and Cummins, 1966; Tyler, Piatigorsky and Ozaki, 1966) and potassium (Tyler and Monroy, 1959) is considerably reduced in unfertilized eggs, as is also the utilization of amino acids until after fertilization (see Monroy, 1965; Tyler and Tyler, 1966b, for reviews).

It has been shown (Tyler, 1949; Tyler and Tyler, 1964a) that sea urchins, after having been induced to spawn by potassium chloride-injection, can produce additional batches of ripe eggs in the laboratory. The yield after ten days to two weeks may often approach the quantity originally obtained. It is, then, possible to label eggs during oogenesis, as has been done in various other animals particularly among the mammals, birds, amphibians, and insects (see Discussion for references), but with the specific advantages that sea urchin eggs provide.

This method was first applied in experiments (Tyler and Hathaway, 1958) to label, with S^{35}, the gelatinous coat (fertilizin) of the egg. Another *in vivo* procedure without preliminary shedding of the eggs consisted of a four-hour incubation of the injected female for labeling of protein (Nakano and Monroy, 1957, 1958; Immers, 1959, 1961; Erb and Maurer, 1962) and of polysaccharide (Immers, 1961). In more recent studies, Gross, Malkin and Hubbard (1965) have again used the longer labeling periods and report effective *in vivo* labeling of RNA with H^3-uridine or P^{32}-phosphate in a period of one week. Also attempts have been made by Holland and Giese (1965) to label the DNA of unfertilized eggs by long periods of maintenance of the injected animals. Only the small oocytes were found to be radioactive in those experiments; but later experiments by Pikó, Tyler and Vinograd (1967) have shown that DNA of ripe eggs can be labeled by the long-term incubation of injected animals in the laboratory.

The present report is for the purpose of demonstrating the effectiveness of the labeling that can be accomplished in sea urchins by the long-term procedure, and of providing some of the parameters for incorporation of C^{14}- and H^3-uridine into the RNA of the ripe unfertilized egg. The results of one experiment of this type have been reported elsewhere (Tyler and Tyler, 1966a, p. 644).

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Materials and Methods

1) *Injection and care of the animals*

Lytechinus pictus and *Strongylocentrotus purpuratus* females were induced to spawn most, or all, of their mature eggs by injecting about 0.5 ml. of 0.55 *M* potassium chloride into their perivisceral cavity. The sea urchins were kept continually moist with artificial sea water in order to prevent injury by dehydration of the external epidermis or the gills (Tyler and Tyler, 1966a). This was accomplished either by immersing the sea urchin in sea water every few minutes or by allowing the animal to shed in a moist chamber. Injections were done with a small hypodermic needle (30-gauge, $\frac{3}{8}$-inch) carefully inserted through the peristome.

One or two days after being shed, the sea urchins were injected with 0.4 to 0.6 ml. of C^{14}- or H^3-uridine in artificial sea water, at concentrations specified in the individual experiments. The equipment for maintaining the animals was that described by Tyler and Tyler (1966a). The sea urchins were placed, in pairs, in covered, transparent plastic boxes (16 cm. × 35 cm.) containing about 1500 ml. of artificial sea water. They were fed eel grass (*Zostera*). Gentle rocking was provided to allow for aeration and circulation of the water, and constant illumination provided so as to supply additional oxygen by the photosynthetic activity of the eel grass. The constant illumination is thought also to retain the animals in gamete-ripening condition. The temperature in the room was kept below the critical limits for the species but high enough to permit reasonably rapid maturation of the gamets; namely 20° C. for *Lytechinus pictus* and 15° to 17° C. for *Strongylocentrotus purpuratus. Lytechinus pictus* proved to be the hardier of the two species. With *Strongylocentrotus purpuratus* there were fewer long-term survivors and thus only a few experiments are reported for this sea urchin. The water was changed approximately every two weeks and new eel grass was added at that time. Algae would grow along the sides of the boxes and this was allowed to remain.

As has been noted elsewhere (Tyler and Tyler, 1966a) sea urchins often die even under, presumably, optimal laboratory conditions present at marine stations. Probably due to the precautions taken in the initial handling of the sea urchins, only few animals died in the present experiments. Sea urchins that survived the first week in the plastic boxes seldom died thereafter even after incubations that lasted longer than one year. It appears, then, that the sea urchins can readily adapt to these conditions if they are not damaged during or after their collection.

2) *Assay of the labeled material in the shed eggs*

At various times after the labeling injection, eggs were obtained from the sea urchins by potassium chloride-induced spawning. The suspensions were screened for oocytes; those containing more than 1% were discarded. This occurred in very few cases.

The assay of radioactivity was performed by the filter-paper procedure of Mans and Novelli (1960) as described previously (Tyler, 1966), the measurements being made in a Packard Tricarb Scintillation Counter with efficiencies of 50% for C^{14} and 2 to 4% for H^3. For this purpose the eggs were washed thoroughly, the gelatinous coat removed by brief exposure to pH 5-sea water, the suspension adjusted to 10.0 ml. and 6 aliquots placed on strips of filter paper and allowed to dry. Two additional aliquots were added to an equal volume of 0.6 *M* potassium hydroxide, incubated at 37° C. for 18 hours and then dried on filter paper strips. Two of the first six filter papers were assayed directly for radioactivity. Four strips, including the two containing the eggs subjected to alkaline hydrolysis, were processed [ice-cold 5% trichloroacetic acid (TCA)] for incorporation of C^{14}- or H^3-uridine into nucleic acid. Finally, the remaining two filter papers were processed [hot (ca. 90° C.) 5% TCA] for incorporation of label into protein, as described elsewhere (Tyler, 1966).

3) *Extraction of RNA*

RNA was extracted from the unfertilized eggs labeled during oogenesis by a procedure quite similar to that utilized by Gross, Malkin and Hubbard (1965). RNA-labeled unfertilized eggs were homogenized in 0.01 *M* sodium acetate, pH 5.0, followed by low-speed centrifugation as given with the results. The supernatant fraction and washed pellet were made to 2% with respect to sodium dodecyl sulfate, to 0.5% with respect to naphthalene disulfonic acid and to 0.3% with respect to purified bentonite. An equal volume of phenol (Mallinckrodt Chemical Co.), supplemented with 0.1% 8-hydroxyquinoline and saturated with 0.01 *M* sodium acetate buffer (pH 5.0), was added to the fractions of the homogenate. The mixture was mechanically shaken at 4° C. for 20 minutes, centrifuged and the phenol phase removed. The phenol extraction was repeated three times, the aqueous phase set aside and the interfacial gel re-extracted with a 0.01 *M* solution of Tris-hydrochloric acid at pH 7.4. The resulting aqueous phase was added to the original one and the RNA was precipitated with 66% (v/v) ethanol and 0.1 *M* sodium chloride overnight at −20° C. The precipitate was washed in absolute alcohol, dried from ether and redissolved in 0.01 *M* potassium acetate, pH 5.2, containing 10^{-3} *M* magnesium chloride and 15 μg./ml. of DNase (Worthington, electrophoretically pure). The solution was incubated at 4° C. for 30 minutes followed by numerous extractions with buffer-saturated phenol at 4° C. until an interface was no longer visible. The aqueous phase was then precipitated as above, washed with absolute alcohol, with ether, air-dried and redissolved in 0.01 *M* sodium acetate buffer, pH 5.0.

Tests with samples of the labeled material thus obtained showed that it became completely soluble in ice-cold 5% TCA after treatment with RNase (50 μg./ml., 37° C. for 30 minutes) or hydrolysis with potassium hydroxide (0.3 *M*, 37° C. for 18 hours) or hot TCA (5%, 90 to 100 ° C. for 15 minutes). The preparations gave 260mμ/280mμ absorption ratios close to 2.

The sedimentation pattern of the labeled RNA was examined by sucrose density-gradient centrifugation under the conditions specified in the section on Results. Sedimentation coefficients have been assigned as approximate values by analogy with those determined under similar conditions by Slater and Spiegelman (1966a) who employed markers of 23S and 16S RNA from *Bacillus megaterium*. The sedimentation coefficients in the present study have also been calculated by the method of Martin and Ames (1961) and these values are in close agreement with those of Slater and Spiegelman (1966a).

RESULTS

1) *Retention of radioactivity by Lytechinus pictus after receiving an injection of H^3-uridine*

To test the retention of radioactively labeled uridine injected into the body cavity of the adult female, the following experiment was performed. Two sea urchins, weighing approximately 15 grams apiece, were each injected with 125 μc. of H^3-5-uridine in 0.5 ml. artificial sea water. They were placed into the same plastic box containing 1500 ml. of artificial sea water but lacking eel grass. The sea urchins were kept rocking gently, as usual, and duplicate 0.5-ml. samples of the medium were removed and assayed for radioactivity at the indicated times. The sea water was not changed for the three-week duration of the experiment. The results are illustrated in Figure 1.

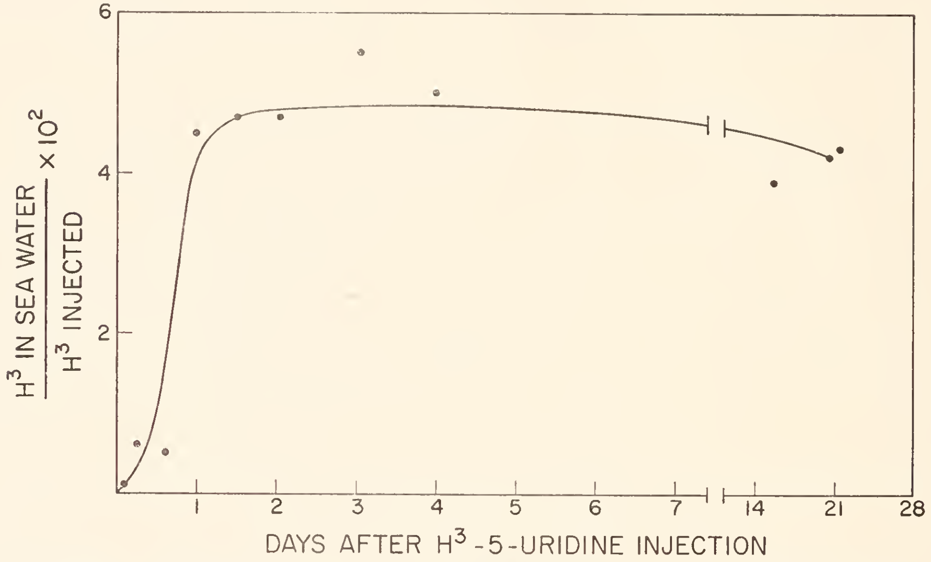

FIGURE 1. Retention of radioactivity by *Lytechinus pictus* after receiving an injection of H^3-5-uridine. Two females were induced to spawn by injection of potassium chloride and then injected with 125 μc. of H^3-5-uridine (sp. act. 25,000 c/M) in 0.5 ml. artificial sea water. They were maintained in the same plastic box (16 cm. × 35 cm.) containing 1500 ml. artificial sea water. Duplicate 0.5-ml. aliquots of the sea water were removed at the specified times and assayed for radioactivity.

For the first 12 hours less than 1% of the injected radioactivity appeared in the medium. By 24 hours, however, almost 5% could be accounted for in the sea water. This value did not increase during the next 21 days. A slight decrease was observed in the third week. It is evident then that sea urchins retain most of the nucleoside introduced into their body cavity under these conditions. It is of interest to note at this time that Erb and Maurer (1962) injected H^3-leucine and H^3-lysine into the coelomic cavity of the sea urchin *Psammechinus miliaris* and observed that only 0.1% of the label escaped from the animal after 4 hours in 45 ml. of sea water.

2) *Uptake and incorporation of C^{14}- and H^3-uridine by maturing oocytes of Lytechinus pictus and Strongylocentrotus purpuratus*

The data obtained from the radioactivity-measurements of the shed, labeled, mature eggs of *Lytechinus pictus* and *Strongylocentrotus purpuratus* are given in Table I. The experiments are grouped according to the type of injected isotope (column 2). They are listed within each group in an order corresponding to increasing time-intervals between injection and collection of the eggs (column 4). The measurements were done in duplicate and both values are reported (columns 6, 7, 8 and 9). The data have been adjusted, for comparative purposes, to relate to 10^{-2} eggs. The actual number of eggs that the animal shed is given in column 5. The data in column 10 list the percentage of the injected label recovered in the shed eggs. The values in column 11 show the percentage of the total label accumulated by the eggs that is incorporated into ice-cold TCA (5%)-insoluble material. Finally, the sensitivity of the incorporated label to acid and alkaline hydrolysis provides information regarding the types of macromolecules into which the label has become incorporated (columns 12 and 13, respectively). Thus, column 12 lists values for the percentage of the macromolecular radioactivity that is nucleic acid, and column 13 lists values for the minimum percentage of the macromolecular radioactivity that is RNA. The latter are minimum values because the potassium hydroxide digestion leaves some protein (50 to 60%) which may be labeled and might account, along with DNA, for some of the radioactivity shown in column 9.

The values listed in column 10 of the table show that the recovery varied considerably in the different tests. This is not surprising since there are many variables that can affect the results of experiments of this type. For instance, among the factors that can be expected to influence the final yield of radioactivity in the shed eggs are (1) seasonal differences of the animals during the time various tests were made, (2) variations in the extent of spawning both before and after administering the radioactive material, (3) possible individual differences in the rate and number of maturing eggs, and (4) differences in the rate at which the label may be utilized by the various tissues of the animal.

In addition there are differences depending upon the particular isotope employed. This can be seen in Figure 2 where the data for per cent recovery at various times after injection are plotted.

For example, the experiments with C^{14}-2-uridine all gave relatively poor recovery. Possibly this may be due to the lower specific radioactivity of C^{14}- than of H^3-uridine, along with saturation of the oocyte's uptake-sites at relatively low concentrations of exogenous uridine. Such saturation of uptake-sites at low concentrations has been found to be true for fertilized sea urchin eggs (Piatigorsky and Whiteley, 1965). The present results with C^{14}-uridine are, then, interpretable in some manner without recourse to the improbable assumption that the C^{14}-labeled uridine is accumulated by the oocyte, and is incorporated into nucleic acid, less readily than the H^3-uridine. Likewise the differences in per cent recovery obtained

TABLE I

Uptake and incorporation of C^{14}- and H^3-uridine by maturing oocytes of the sea urchins *Lytechinus pictus* and *Strongylocentrotus purpuratus* during long-term *in vivo* labeling of spawned females. Each animal received only one injection and was maintained under the conditions specified in Materials and Methods

(1) Expt. No.*	(2) Isotope (c/M)	(3) μc. injected	(4) Days labeled	(5) No. of eggs shed	(6) cpm per 10^2 eggs** No TCA	(7) Cold TCA	(8) Hot TCA	(9) KOH	(10) % recovery of injected label	(11) % label in macromolecules***	(12) % label in nucleic acids relative to label in macromolecules****	(13) Minimum % label in RNA relative to label in macromolecules*****
1	C^{14}-2-uridine (25.2)	10	3	5.0×10^4	231	34	13	—	1.10	14	60	—
					256	33	14					
2	C^{14}-2-uridine (25.2)	10	8	8.0×10^5	20	8.7	—	—	1.47	43	—	—
					20	8.6						
3	C^{14}-2-uridine (25.2)	10	9	1.5×10^5	7.9	1.4	—	—	0.13	17	—	—
					12	1.9						
4	C^{14}-2-uridine (25.2)	10	15	3.0×10^5	49	26	14	—	1.32	56	41	—
					48	28	18					
5	C^{14}-2-uridine (25.2)	10	20	8.0×10^5	12	3.0	0.3	—	0.84	25	90	—
					12	3.1	0.3					
6	C^{14}-2-uridine (25.2)	10	24	1.3×10^5	7.0	1.1	0.2	—	0.09	16	86	—
					6.8	1.1	0.1					
7	C^{14}-2-uridine (25.2)	10	28	8.2×10^4	28	4.2	0.3	—	0.21	14	91	—
					30	3.7	0.4					
8	H^3-6-uridine (6550)	100	7	3.8×10^4	230	77	12	—	2.00	34	86	—
					225	76	10					
9	H^3-6-uridine (6550)	100	9	4.6×10^4	17	10	2.1	—	0.16	60	75	—
					13	8.0	2.4					
10	H^3-6-uridine (6550)	100	10	8.8×10^4	439	268	25	—	8.57	67	92	—
					418	302	18					

* Experiments 1–22, *Lytechinus pictus*; experiments 23–28, *Strongylocentrotus purpuratus*.

** 0.005 or 0.01 of the shed eggs were assayed for each determination. The values represent the cpm's left on the filter papers after the indicated treatments as described under Materials and Methods (No TCA = total uptake; Cold TCA = protein and nucleic acid; Hot TCA = protein; KOH = DNA and some protein).

*** $\left[\frac{\text{column (7)}}{\text{column (6)}}\right] \times 100.$

**** $\left[\frac{\text{column (7)} - \text{column (8)}}{\text{column (7)}}\right] \times 100.$

***** $\left[\frac{\text{column (7)} - \text{column (9)}}{\text{column (7)}}\right] \times 100 \times \text{column (12)}.$

****** Uniformly labeled H^3-uridine (experiments 23–28).

TABLE 1—(*Continued*)

(1) Expt. No.*	(2) Isotope (c/M)	(3) μc. injected	(4) Days labeled	(5) No. of eggs shed	(6) cpm per 10^2 eggs** No TCA	(7) Cold TCA	(8) Hot TCA	(9) KOH	(10) % recovery of injected label	(11) % label in macromolecules***	(12) % label in nucleic acids relative to label in macromolecules****	(13) Minimum % label in RNA relative to label in macromolecules*****
11	H^3-6-uridine	100	13	1.5×10^5	132	51	19	—	4.06	46	65	—
	(6550)				107	59	20					
12	H^3-6-uridine	150	15	5.0×10^5	210	144	110	—	16.5	67	32	—
	(6550)				227	148	89					
13	H^3-6-uridine	100	22	8.0×10^4	5.2	3.1	1.9	—	2.35	72	35	—
	(6550)				5.1	4.3	2.9					
14	H^3-6-uridine	150	28	3.0×10^5	434	389	14	5.5	19.6	93	97	96
	(6550)				427	408	11	4.3				
15	H^3-5-uridine	150	25	7.7×10^5	193	143	9.3	4.8	22.5	74	94	90
	(25,000)				192	141	9.0	6.4				
16	H^3-5-uridine	120	33	2.0×10^5	592	691	20	14	24.5	103	97	95
	(25,000)				703	649	24	10				
17	H^3-5-uridine	150	34	5.3×10^5	323	318	19	10	24.4	117	94	91
	(25,000)				279	387	21	10				
18	H^3-5-uridine	120	36	6.4×10^5	76	68	5.8	2.0	9.44	88	90	87
	(20,000)				80	70	7.4	2.8				
19	H^3-5-uridine	120	40	2.3×10^5	741	575	36	13	32.0	78	94	92
	(20,000)				731	571	35	14				
20	H^3-5-uridine	120	61	2.0×10^5	321	293	28	—	12.1	85	90	—
	(20,000)				319	254	25					
21	H^3-5-uridine	125	64	6.4×10^5	192	188	13	9.4	23.6	87	91	86
	(20,000)				196	149	16	9.4				
22	H^3-5-uridine	120	89	6.4×10^5	76	67	5.7	2.0	9.44	88	90	87
	(20,000)				80	70	7.4	2.8				
23	H^3-uridine******	100	13	2.0×10^5	243	156	19	—	10.7	66	87	—
	(20,000)				230	154	20					
24	H^3-uridine	125	14	4.9×10^6	2.9	2.0	0.6	—	2.40	75	71	—
	(20,000)				2.6	2.1	0.6					
25	H^3-uridine	125	14	1.7×10^6	4.4	2.6	1.0	—	1.28	73	68	—
	(20,000)				3.8	3.4	0.9					
26	H^3-uridine	125	14	7.4×10^5	25	21	4.7	—	3.51	81	77	—
	(20,000)				27	21	5.0					
27	H^3-uridine	100	16	1.5×10^6	0.6	1.0	0.1	—	0.20	108	85	—
	(20,000)				0.6	0.3	0.1					
28	H^3-uridine	100	16	2.3×10^5	45	36	4.7	—	2.29	78	86	—
	(20,000)				44	33	5.2					

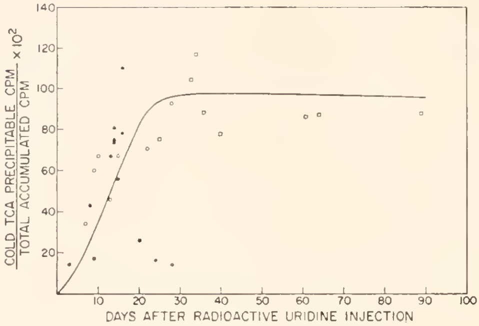

FIGURE 2. Percentage recovery of injected isotope found in mature eggs of *Strongylocentrotus purpuratus* and *Lytechinus pictus* labeled during oogenesis. Sea urchins were spawned with potassium chloride, injected with labeled uridine and incubated at 20° C. as given in Materials and Methods. At the designated time after the injection of radioactive uridine, the mature eggs were shed with potassium chloride and assayed for total radioactivity by scintillation counting. Each point on the figure specifies the percentage of radioactivity, relative to that administered to the animal, that was recovered in the shed eggs. ●—*L. pictus*, C^{14}-2-uridine. ○—*L. pictus*, H^3-6-uridine. □—*L. pictus*, H^3-5-uridine. ▲—*S. purpuratus*, H^3-uniformly labeled-uridine.

with the two H^3-uridines (labeled in the 5 or the 6 positions) that were employed in the experiments with *L. pictus* may be similarly interpreted.

When expressed in terms of the amounts of uridine taken up by the oocytes in the various experiments the values for uptake of C^{14}-uridine are as high, or higher, than those obtained with H^3-uridine. For *L. pictus* values for the uptake of C^{14}-2-uridine per 10^2 eggs range from 0.25 (experiment 6) to 8.73 (experiment 1) $\mu\mu$moles while those of H^3-6-uridine are 0.01 (experiment 13) to 0.99 (experiments

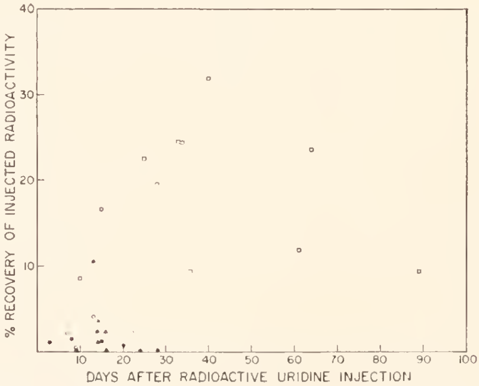

FIGURE 3. Percentage of radioactivity precipitable with ice-cold trichloroacetic acid in the shed eggs of *Strongylocentrotus purpuratus* and *Lytechinus pictus* labeled during oogenesis. The percentage of radioactivity precipitable with ice-cold 5% trichloroacetic acid, relative to the total radioactivity accumulated by the shed eggs, has been plotted from the values listed in Table I. ●—*L. pictus*, C^{14}-2-uridine. ○—*L. pictus*, H^3-6-uridine. □—*L. pictus*, H^3-5-uridine. ▲—*S. purpuratus*, H^3-uniformly labeled-uridine.

10 and 14) $\mu\mu$moles and those for H^3-5-uridine are 0.05 (experiments 18 and 22) to 0.39 (experiment 16) $\mu\mu$moles.

Overall, the data for uptake as a function of time after injection show progressive increase during a period of one month. For the experiments of one month or longer there is an average recovery of some 20% of the injected material.

Values for the per cent of the total radioactivity present in the shed eggs that is incorporated into cold-acid-insoluble material are plotted in Figure 3. A plateau of about 95% is reached one month after injection.

Hence, even though these parameters are strongly influenced by factors, mentioned above, that are difficult to control, it is feasible to estimate the order of magnitude of uptake and incorporation of uridine that may be expected to appear in the eggs when sea urchins are maintained for various lengths of time under these conditions. From the present results it appears that the minimum time necessary to achieve maximum labeling of unfertilized eggs is approximately one month, if only one injection of labeled uridine is given to the animal.

The ratios of the radioactivity precipitable with cold 5% TCA, after acid and alkaline hydrolysis of the labeled eggs, to that precipitable before hydrolysis, give an index of the proportion of label that has become incorporated into total nucleic acid and RNA, respectively. Hot acid (5% TCA, 90 to 100° C. for 15 minutes) will hydrolyze nucleic acid but not protein, while dilute alkali (0.3 *M* potassium hydroxide at 37° C. for 18 hours) will degrade RNA and some protein but not DNA (Davidson, 1965). The data in column 12 of Table I show that much of the labeled uridine was incorporated into nucleic acid. The precipitable radioactivity, however, was not completely removed by the hot acid hydrolysis, indicating that some label has become incorporated into protein. The degree of specificity of incorporation of the label into nucleic acid was different when the injected uridine was labeled in different positions of the molecule. The average percentages of labeled material sensitive to acid hydrolysis, when the precursors were C^{14}-2-uridine (experiments 1–7), H^3-6-uridine (experiments 8–14), H^3-5-uridine (experiments 15–22) or uniformly labeled H^3-uridine (experiments 23–28) were 74%, 69%, 93% and 79%, respectively. Thus H^3-5-uridine gave the highest specific incorporation into nucleic acid.

As noted above, column 13 of Table I lists values for the percentage of the label of the macromolecules that is in RNA. It is evident from these figures that the radioactivity of the H^3-5-uridine is incorporated mostly (about 90%) into RNA.

3) *Sedimentation pattern of the RNA labeled during maturation of Lytechinus pictus oocytes*

The radioactive RNA of the ripe eggs that had been labeled for 33 days during oogenesis was phenol-extracted from homogenates, and fractions thereof, and analyzed by sucrose density-gradient centrifugation. Figure 4 shows the sedimentation pattern of the labeled RNA obtained from the 10,000 *g* supernatant fraction (Fig. 4A) and from the corresponding pellet (Fig. 4B).

The solid lines of the figures trace the absorbancies at 260 mμ of the successive fractions of the sucrose gradients and show the three predominant species of RNA with their characteristic peaks at approximately 28S, 18S and 4S. The dashed

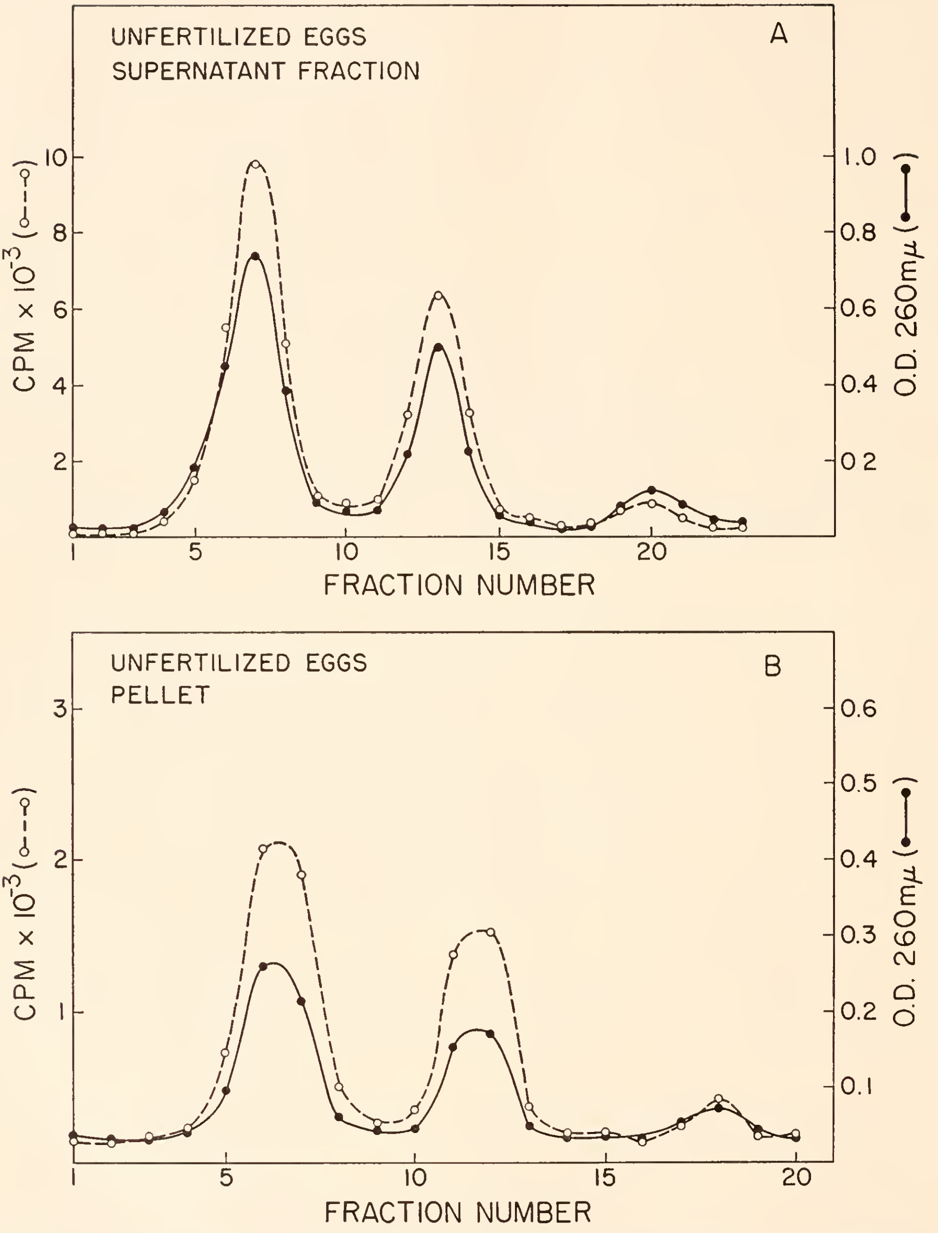

FIGURE 4. Sedimentation pattern of RNA from the supernatant fraction (A) and the mitochondrial pellet (B) of an homogenate of RNA-labeled unfertilized eggs of *Lytechinus pictus.* A spawned female was injected with 150 μc. of H^3-5-uridine (sp. act. 25,000 c/M) and shed 33 days later (experiment 16 of Table I). The labeled unfertilized eggs were washed several times by centrifugation in 0.55 *M* KCl, homogenized in 3 volumes of 0.01 *M* Na acetate buffer at pH 5.0, centrifuged at 10,000 *g* for 10 minutes and the supernatant fraction set aside at 0° C. The pellet was washed twice by centrifugation with 20 to 30 volumes of homogenization

lines give the values for the radioactivity of the same fractions and show a similar pattern. Both the supernatant fraction and the pellet contain the same types of RNA and in approximately the same relative amounts. In addition to the main components there are smaller amounts of heterogeneously sedimenting labeled RNA that appear in these profiles, as well as in others from extracts of whole homogenates. These are evident in the regions of the graphs outside the areas covered by the three main components. Presumably this represents messenger RNA which may be present also in the areas covered by the principal RNA components.

The distribution of label among the various RNAs has been estimated by a method described by Girard, Latham, Penman and Darnell (1965) which involves the assumption that the areas under the 28S and 18S regions include heterogeneously sedimenting RNA at the same level as outside these regions. Thus, the total amount of messenger RNA is obtained by a summation of the radioactivity under a baseline extending to the 4S region. The baseline is drawn at a level corresponding to the average radioactivity outside the 28S and 18S regions. Subtraction of the appropriate values from the cpm's in the 28S and 18S regions gives the amounts of label assigned to the ribosomal RNAs. This procedure gives the following percentages of label in the various types of RNA for the experiment shown in Figure 4:

28S = 49% ; 18S = 29% ; heterogeneous = 15% ; 4S = 6% (Supernatant)
28S = 42% ; 18S = 28% ; heterogeneous = 21% ; 4S = 8% (Pellet)
28S = 48% ; 18S = 29% ; heterogeneous = 16% ; 4S = 7% (Total)

In another test of this type in which the RNA was extracted from a whole homogenate of eggs, labeled with H^3-5-uridine for 64 days (experiment 21 of Table I), the distribution of radioactivity was:

28S = 48% ; 18S = 27% ; heterogeneous = 21% ; 4S = 5% (Total)

It can be concluded, then, that most of the label incorporated into RNA by the oocytes resides in the two species of ribosomal RNA. Much less radioactivity is incorporated into transfer RNA and an upper limit of 10 to 20% can be placed on the incorporation of H^3-uridine into heterogeneously sedimenting messenger RNA.

DISCUSSION

The results of this study have shown that the RNA of ripe eggs of sea urchins can be labeled, at high specific activity, by maintaining the animals in relatively small volumes of sea water for prolonged periods after a single injection of radioactive nucleoside. About 95% of the injected isotope is retained by the sea urchin. From the measurements with animals starting one month after injection, an average of 20% with a maximum of 32% was recovered in the eggs. In most of the

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buffer, all supernatant fractions combined and the pellet resuspended in about 40 volumes of buffer. The preparations were phenol-extracted at 4° C. and treated with DNase as described in Materials and Methods. Samples (0.3 ml.) of the labeled extracts were centrifuged through a linear 5 to 20% sucrose density-gradient (in 0.01 M Na acetate and 0.1 M NaCl at pH 5.0) at 37,000 rpm for 5 hours at 5 to 10° C. Three-drop (about 0.20-ml.) fractions were collected after bottom puncture of the centrifuge tube. These were diluted with an equal volume of distilled water and the 260 mμ absorption was determined in each fraction. Measurements of radioactivity were then made on the same samples by scintillation counting as given in Materials and Methods .

experiments, almost all of the label appeared in macromolecules, principally RNA. The isotope which gave the most consistently high recovery and specific incorporation into RNA was H^3-5-uridine. The shortest time for optimum RNA-labeling of unfertilized eggs was approximately one month under the present conditions.

In eggs obtained at various times less than one month after injection proportionately more of the label was found in low molecular weight (acid-soluble) materials. Evidently, then, the injected uridine can be retained by the eggs in some form in which it can be later incorporated into RNA. If the situation is analogous to that in fertilized eggs, as explored in the experiments of Piatigorsky and Whiteley (1965), it may be concluded that the uridine is stored as nucleoside triphosphates.

As noted in the introduction, *in vivo* incorporation of radioactive materials into oocytes has been accomplished in many different types of animals, among them being mammals (Sirlin and Edwards, 1959; Rudkin and Griech, 1962), chickens (Hevesy and Hahn, 1938; Chargaff, 1942; Patterson, 1961), amphibians (Ficq, 1955, 1961, 1966; Brachet and Ficq, 1956; Gall and Callan, 1962; Davidson, Allfrey and Mirsky, 1964; Brown and Littna, 1964a, 1964b; Davidson, Crippa, Kramer and Mirsky, 1966), insects (Sirlin and Jacob, 1960; Favard-Séréno and Durand, 1963a, 1963b; Bier, 1963; Zalokar, 1965) and sea urchins (Tyler and Hathaway, 1958; Gross, Malkin and Hubbard, 1965; Holland and Giese, 1965; Pikó, Tyler and Vinograd, 1967). In some organisms, notably chickens and insects (see Tyler, 1955, and Williams, 1965, for reviews), growth of the oocyte is associated with the accumulation of materials synthesized in other cells of the body. This may occur to some extent in all animals. However, at least for amphibians (Izawa, Allfrey and Mirsky, 1963), sea urchins (Piatigorsky, Ozaki and Tyler, 1967), and marine polychaete worms (Tweedell, 1966) evidence has been provided that immature oocytes isolated from the ovary are capable of intense RNA and protein synthesis. It is known also that mature sea urchin eggs while in the ovary incorporate little, if any, labeled precursors into RNA (Immers, 1961; Ficq, 1964; Gross, Malkin and Hubbard, 1965). Thus, the labeled RNA of the shed eggs in the present experiments can be assumed to have been synthesized primarily by the oocytes themselves during oogenesis.

In vivo RNA-labeling experiments (Brown and Littna, 1964b; Davidson, Allfrey and Mirsky, 1964) with the toad *Xenopus laevis* have shown that growing oocytes synthesize predominantly 28S and 18S ribosomal RNA. Much less 4S RNA was synthesized by oocytes in their tests. The most active time of RNA synthesis was found to occur during the lampbrush phase of oocyte growth (Davidson, Allfrey and Mirsky, 1964). Some non-ribosomal RNA that sediments heterogeneously in a sucrose density-gradient was also shown to be synthesized during oogenesis (Brown and Littna, 1964a; Davidson, Allfrey and Mirsky, 1964). The labeled ribosomal and heterogeneously sedimenting RNAs were conserved throughout oogenesis and during early development of the fertilized egg.

Davidson, Crippa, Kramer and Mirsky (1966) showed that RNA extracted from lampbrush stage oocytes of *Xenopus* possesses considerable capacity to stimulate the *in vitro* incorporation of labeled amino acids into protein. Hybridization studies indicated that about 1.5% of homologous DNA could be bound with RNA, labeled *in vivo,* extracted from lampbrush phase oocytes. Furthermore, since unlabeled RNA from later stage, mature oocytes competed with the labeled RNA from lampbrush stage oocytes for hybridization with homologous DNA, it was concluded that the RNA synthesized throughout oogenesis is conserved and sequestered in the mature oocyte.

Gross, Malkin and Hubbard (1965) investigated *in vivo* RNA synthesis by oocytes of the sea urchin *Arbacia punctulata* during their final week of maturation. They showed that in sea urchins, too, growing oocytes synthesize primarily 28S and 18S ribosomal RNA and that the labeled RNAs are preserved in the mature egg. Apart from labeled ribosomal RNA, Gross, Malkin and Hubbard (1965) showed that some 4S RNA becomes labeled during oogenesis. In addition they found small quantities of labeled RNA of higher specific radioactivity than the ribosomal RNA. This labeled RNA sedimented heterogeneously in a sucrose density-gradient and was eluted from a methylated albumin-kieselguhr column at higher ionic strength than was the labeled ribosomal RNA. Furthermore, about 1.5% of the labeled RNA phenol-extracted from unfertilized eggs hybridized with homologous DNA even in the presence of a 350% excess of non-radioactive ribosomal RNA.

The present experiments, as well as others to be presented elsewhere (Piatigorsky, in preparation), are in accord with those cited above with respect to the relatively larger amounts of 28S and 18S RNA than of 4S and heterogeneously sedimenting RNAs that are made by the growing oocytes. These labeled RNAs are conserved in mature unfertilized eggs for prolonged periods of time. The intense labeling of the oocyte nucleolus with labeled RNA-precursors in sea urchins (Ficq, 1964; Piatigorsky, Ozaki and Tyler, 1967), starfish (Ficq, 1953, 1955; Vincent, 1954), amphibians, Ficq, 1961, 1964; Ozban, Tandler and Sirlin, 1964), polychaete worms (Tweedell, 1966) and some insects (Zalokar, 1965) is consistent with large quantities of ribosomal RNA being synthetized by the nucleolus (Perry, 1965) of the oocyte.

The present tests show that the incorporation of H^3-uridine into heterogeneously sedimenting RNA does not exceed one-tenth to one-fifth of the total. This value is based on the assumption that labeled messenger RNAs are present in the 28S and 18S regions at the same level as outside these regions. Gross, Malkin and Hubbard (1965) utilized a comparable procedure to estimate a maximum value of label incorporated into heterogeneously sedimenting RNA during the final stages of oogenesis in sea urchins. Their determinations indicated that 10 to 15% of the total label sedimented heterogeneously. These percentages of label in the various types of RNA do not necessarily reflect mass ratios since consideration of possible specific radioactivity differences have been neglected. Nevertheless, it would seem that the 10 to 20% of heterogeneously sedimenting radioactivity in the extracted RNAs would easily account for the 4 to 5% of the total RNA of unfertilized eggs possessing template potential with respect to the *in vitro* incorporation of labeled amino acids into protein (Slater and Spiegelman, 1966b).

Summary

The present experiments provide data on the results of labeling the RNA of sea urchin eggs during oogenesis, by injection of C^{14}- and H^3-uridine into the perivisceral cavity of previously spawned females. Not more than 5% of the label was

found in the surrounding sea water during the first three weeks after injection. Mature eggs were obtained from animals kept for various periods of time extending to three months after injection. Optimum labeling of the RNA generally occurred at one month, at which time the eggs contained on the average some 20% of the injected label of which 95%, on the average, was in the form of macromolecules. Additional assessment, in eight of the 28 experiments of the percentage of the cold-acid-precipitable label that was in RNA gave minimum values ranging from 86 to 96%. Sucrose density-gradient centrifugation profiles of the extracted RNA showed the label to be mostly (70 to 80%) ribosomal, with about 1.5 times as much 28S as 18S RNA, and about 5 to 10% transfer RNA (4S). The heterogeneously sedimenting labeled RNA, possibly messenger RNA, would amount to an upper limit of 10 to 20% of the total.

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NA$^+$- AND K$^+$-STIMULATED RESPIRATION IN THE EXCISED GILL OF THE LAND CRAB, CARDISOMA GUANHUMI[1]

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The mechanisms by which various components of biological membranes participate in active ion transport and share in other aspects of membrane permeability are of central significance in biological regulation. A Na$^+$- and K$^+$-stimulated ATPase enzyme system has been described in crab nerve (Skou, 1957) and suggested as the enzymatic basis for Na$^+$ and K$^+$ transport. This enzyme complex has been characterized in a variety of tissues (Bonting *et al.*, 1961; Deul and McIlwain, 1961; Post *et al.*, 1960; Whittam, 1962; Glynn, 1962) and recently has been the subject of comprehensive reviews (Judah and Ahmed, 1964; Skou, 1965). The location of the Na$^+$- and K$^+$-stimulated ATPase activity in some component of the cell membrane (Post *et al.*, 1960; Järnefelt, 1961) and the requirement of this system for respiratory energy (Hyman, 1966; Samson and Quinn, 1967; Martin and Diamond, 1966) suggest that ionic regulation in the intact animal may be energy-dependent.

In decapod Crustacea the lamellar epithelium of the gill has been suggested as the principal site of ion uptake (Dehnel and Carefoot, 1965). Earlier studies (Koch *et al.*, 1954; Gross, 1957) showed that the gills and the epithelium lining the gill chambers were important sites of exchange of ions and water. We have described a Na$^+$- and K$^+$-stimulated ATPase activity (NaKATPase) from gill homogenates of the land crab, *Cardisoma guanhumi,* and have indicated that this enzyme system participates in ionic regulation (Quinn and Lane, 1966).

The elevated Q_{O_2} of excised decapod gill tissue suspended in hypo-osmotic media has been attributed to increased osmotic work (Remane and Schlieper, 1958; Dehnel and McCaughran, 1964). The Q_{O_2} of excised gill tissue from *C. guanhumi* increased in hypo-osmotic media and was stimulated specifically by Na$^+$ and K$^+$. The Q_{O_2} of excised gill tissue measured in 75% sea water, Na$^+$ alone, or Na$^+$ + K$^+$ alone was inhibited by ouabain. The present study suggests that some of the energy liberated by tissue respiration is utilized by the gill NaKATPase enzyme complex in maintaining ionic concentration gradients in the intact animal.

MATERIALS AND METHODS

Specimens of *C. guanhumi,* captured locally, were maintained in the laboratory in shallow trays provided with running sea water and fresh water. The crabs were given laboratory rat chow occasionally and appeared to be in good condition for several months. During the experiments, individual crabs were maintained in 2 liters of varying dilutions of sea water. Those animals providing gill tissue for respiration measurements were moved through several changes of tap water or full strength sea water during the two weeks preceding their use.

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[2] Postdoctoral Fellow.

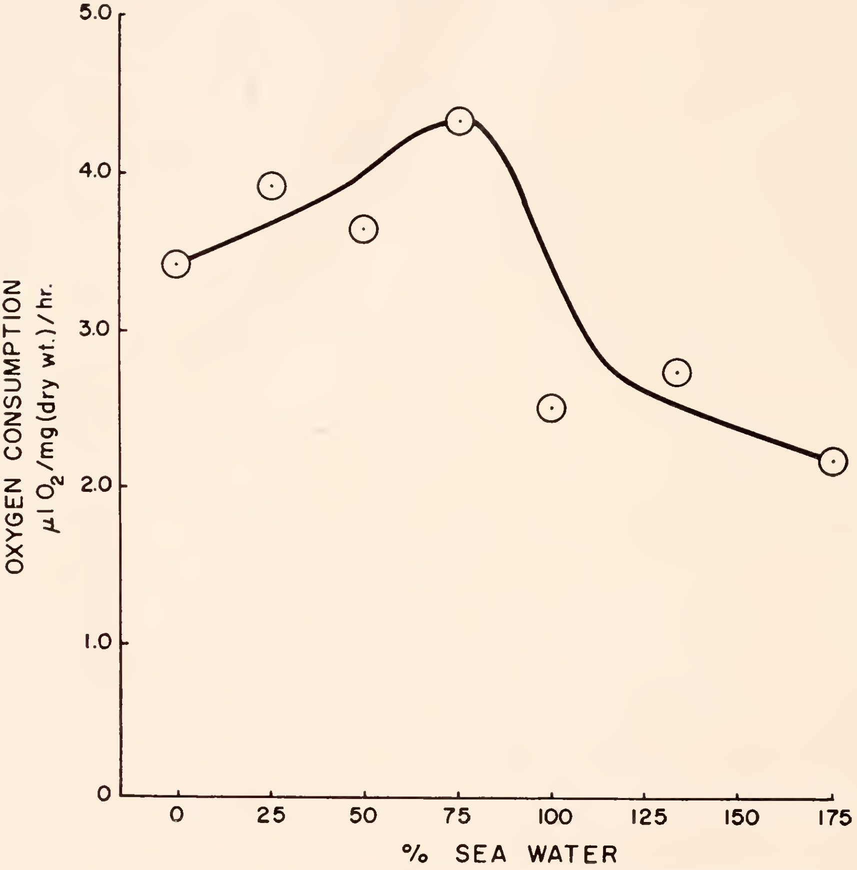


FIGURE 1. Effect of salinity on the respiration of excised gill. Individual crabs were held in 2 l. of 100% sea water for at least 2 weeks prior to each experiment. Each point is an average value derived from 3 to 11 different crabs.

The oxygen consumption of excised gills was determined by the direct method of Warburg. Individual gills (approximately 100 mg. wet weight) were removed intact and placed in a pH 7.0 rinse solution containing 50 m*M*/l. of Tris (hydroxymethyl) amino methane, (Tris) where each gill was cut into 3 or 4 pieces. The gill pieces were washed a second time with fresh buffer and preincubated at room temperature (5–10 minutes) in beakers containing media in which the oxygen consumption was to be measured. Conventional Warburg flasks containing 2.0 ml. of medium were loaded with 3 pieces of gill (8–12 mg. dry wt.). Endogenous Na^+

contributed 2–4 mM/l. and K^+ 1–2 mM/l. to the reaction mixture. The gas phase was atmospheric air and the bath temperature was 27° C. In some experiments the incubation medium consisted of various salinities of sea water, prepared by adding glass-distilled water to a stock solution of 70‰ salinity sea water, prepared by evaporation at ambient temperature. The final salinity was determined with a refractometer. In other experiments the medium was buffered with 15 mM/l. Tris (pH 7.0) in the presence of 10 mM/l. glucose (final concentrations). Values obtained from flasks containing Tris buffer and glucose did not differ appreciably from those obtained using pure sea water. Dry weight of tissue was determined after the washed gill pieces had been dried at 95–105° C. for 24 hours. Preparation of the gill for study generally required about 20 minutes. After a 30–40-minute temperature equilibration period, oxygen uptake was measured for 50 minutes, during which time it was linear.

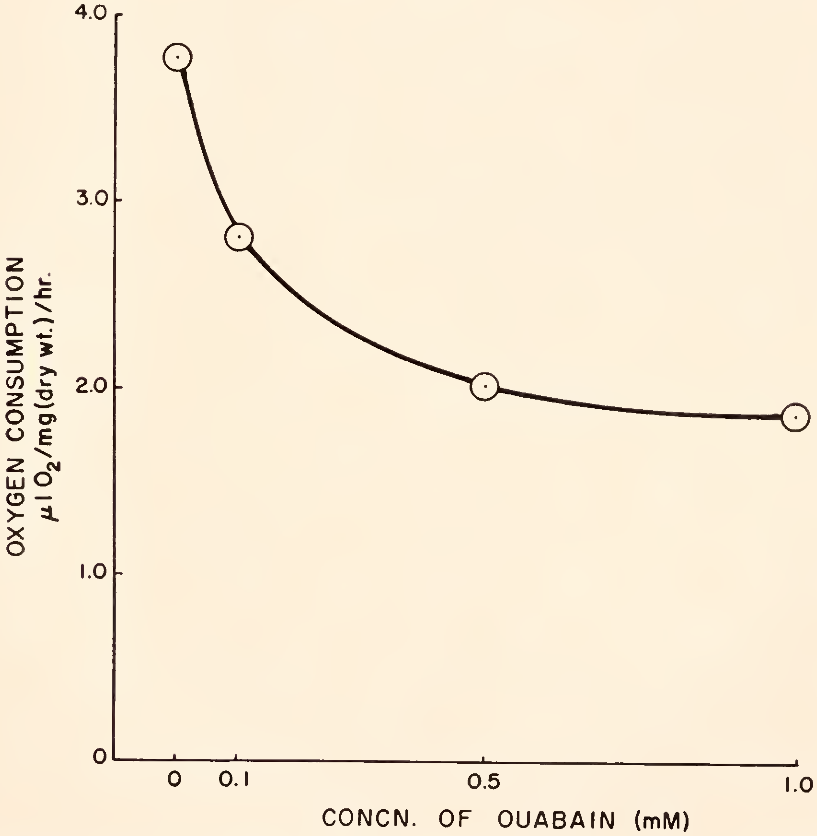


FIGURE 2. Effect of ouabain on excised gill respiration in 75% sea water. Individual crabs were maintained in 2 l. of 100% sea water for 2 weeks prior to their use. The final concentrations of ouabain are indicated on the graph. Each point is an average derived from up to 10 different crabs.

Results and Discussion

The oxygen consumption of Crustacea is influenced by osmotic stress and varies with the ionic regulatory ability of the species studied. For example, in *Eriocheir sinensis* there is no change in oxygen consumption when whole animals are studied in fresh water and in full strength sea water (Schwabe, 1933). In stenohaline *Maja* and *Libinia* the Q_{O_2} in 50% sea water is significantly decreased from that in 100% sea water (King, 1965). In euryhaline *Carcinus* and *Callinectes* oxygen consumption is increased 30–50% in dilute sea water (King, 1965). In *Hemigrapsus nudus* and *H. oregonensis* oxygen consumption is increased as the animals are exposed to increasingly dilute sea water (Dehnel, 1960). This increase in Q_{O_2} is attributed to work done in maintaining an osmotic gradient between the blood and external medium (Dehnel and McCaughran, 1964).

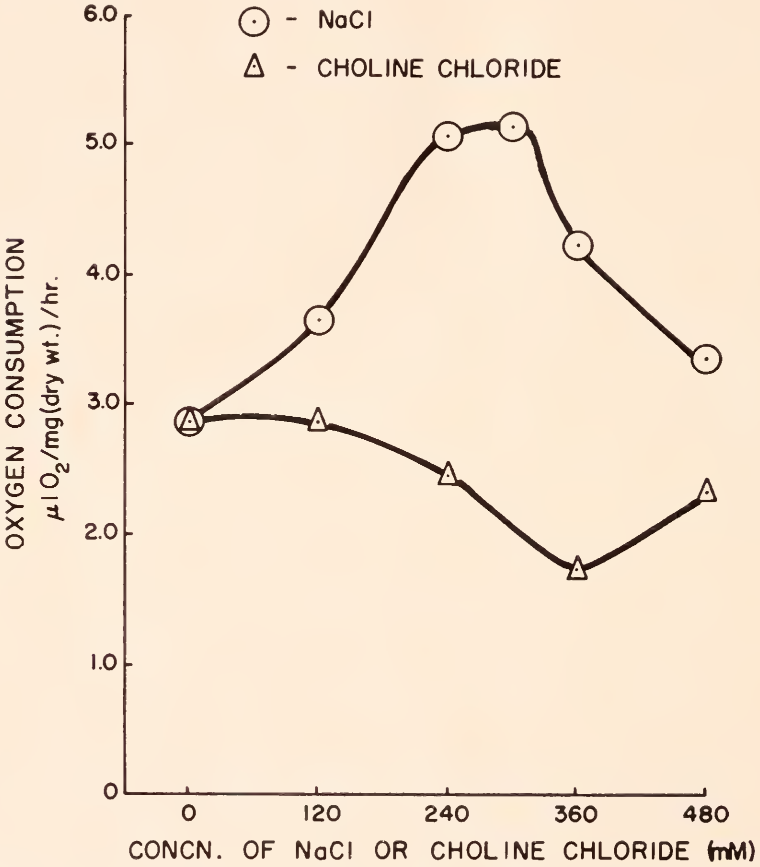


FIGURE 3. Effect of NaCl and choline chloride on oxygen uptake by excised gill. Individual crabs were maintained in 2 l. of tap H_2O for 2 weeks prior to the experiments. Each point is an average value of up to 16 different crabs. The range of final sodium concentrations on the graph correspond to 0–100% sea water.

The oxygen consumption of excised *C. guanhumi* gill tissue (Fig. 1) is higher in dilute sea water than in full strength or concentrated sea water. The elevated Q_{O_2} of excised *C. guanhumi* gill segments in dilute sea water is similar to the increased gill Q_{O_2} in *Callinectes* (King, 1965) where the Q_{O_2} of isolated gills measured in 50% sea water increased 10–30% over that in 100% sea water. In *C. guanhumi* maximum Q_{O_2} of gill tissue occurs in 75% sea water (Fig. 1), suggesting that this concentration of ions is optimal for isolated gill tissue respiration. It may be noted that Na^+ and K^+ concentrations in the blood of *C. guanhumi* are isosmotic with 75% sea water (Quinn and Lane, 1966; Lane, Quinn, and Gifford, unpublished results). We attribute the decrease in Q_{O_2} of gill tissues immersed in either more concentrated or more dilute media to diminished activity of ion pumps when the ionic composition of the extracellular fluids is abnormal. Alternatively the decrease in oxygen consumption may reflect a breakdown of the ion pumps as a consequence of osmotic disruption of the tissue. Indeed, the lowest gill oxygen consumption occurred in hyperosmotic media.

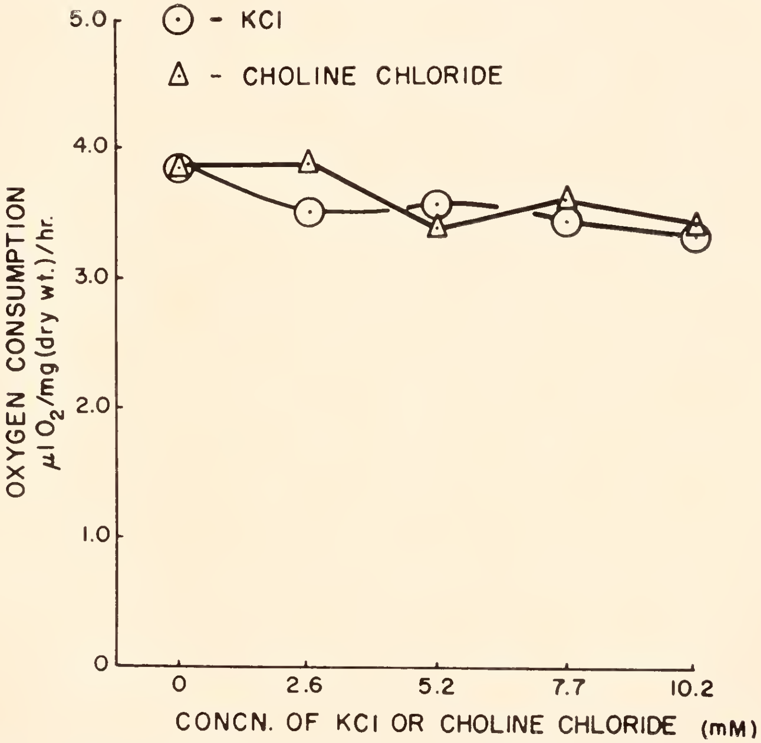


FIGURE 4. Effect of KCl and choline chloride on excised gill respiration. Procedure was similar to that in Figure 3, except that KCl was substituted for NaCl. Each point is the average of up to 6 different crabs.

The cardiac glycoside ouabain inhibits Na^+ transport in frog skin (Kofoed-Johnson, 1958), in red blood cells (Glynn, 1957; Whittam, 1958), and in squid axon (Caldwell and Keynes, 1959). Ouabain specifically inhibits the NaKATPase activity isolated from red blood cells (Post *et al.*, 1960; Dunham and Glynn, 1961), crab nerve (Skou, 1957), gill tissue homogenates of *C. guanhumi* (Quinn and Lane, 1966), and other tissues (Bonting *et al.*, 1961; Whittam, 1962; Glynn, 1963). Brain mitochondrial respiration measured in the presence of brain microsomes and ouabain is reduced by about 40%, due presumably to the inhibition by ouabain of the NaKATPase activity contained in the microsomes (Whittam and Blond, 1964).

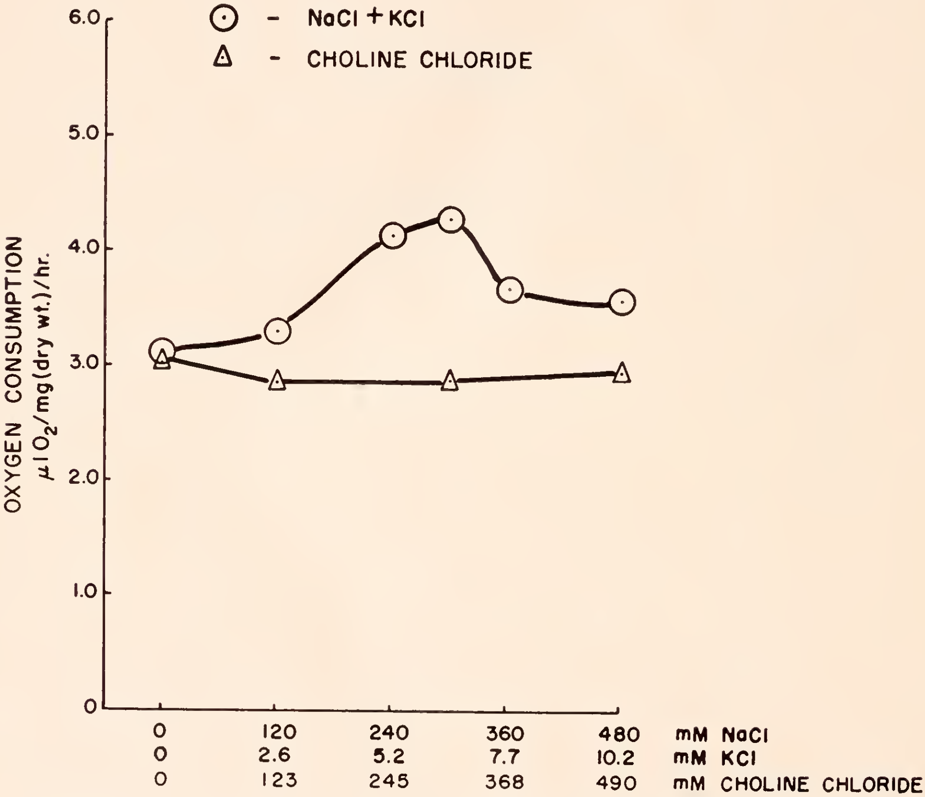


FIGURE 5. Effect of NaCl + KCl, and choline chloride on excised gill respiration. Procedure is the same as for previous figures, except that NaCl and KCl were added together. Each point is an average derived from 18 different crabs.

In addition, the fraction of the total oxygen consumption which is stimulated by $Na^+ + K^+$ in rat brain homogenates (Samson and Quinn, 1967) and in the gall bladder of the rabbit (Martin and Diamond, 1966) is inhibited by ouabain, indicating that a large percentage of respiratory energy flows through the NaKATPase systems in these tissues. Similarly, the Q_{O_2} of excised gills of *C. guanhumi* recorded in 75% sea water was reduced by low concentrations of ouabain (Fig. 2) to less than the level attained by osmotic disruption of the tissue in 175% sea water (Fig. 1).

Since Na^+ is the major ion in sea water and is actively transported across many biological membranes, the effect of Na^+ on oxygen consumption was studied in excised gills. At 310 m*M*/l. Na^+ the excised gill Q_{O_2} was maximally stimulated (Fig. 3) while equivalent osmolar concentrations of choline chloride resulted in a decrease in oxygen consumption. These results indicate that neither osmotic effects nor chloride ions are responsible for the increased oxygen consumption.

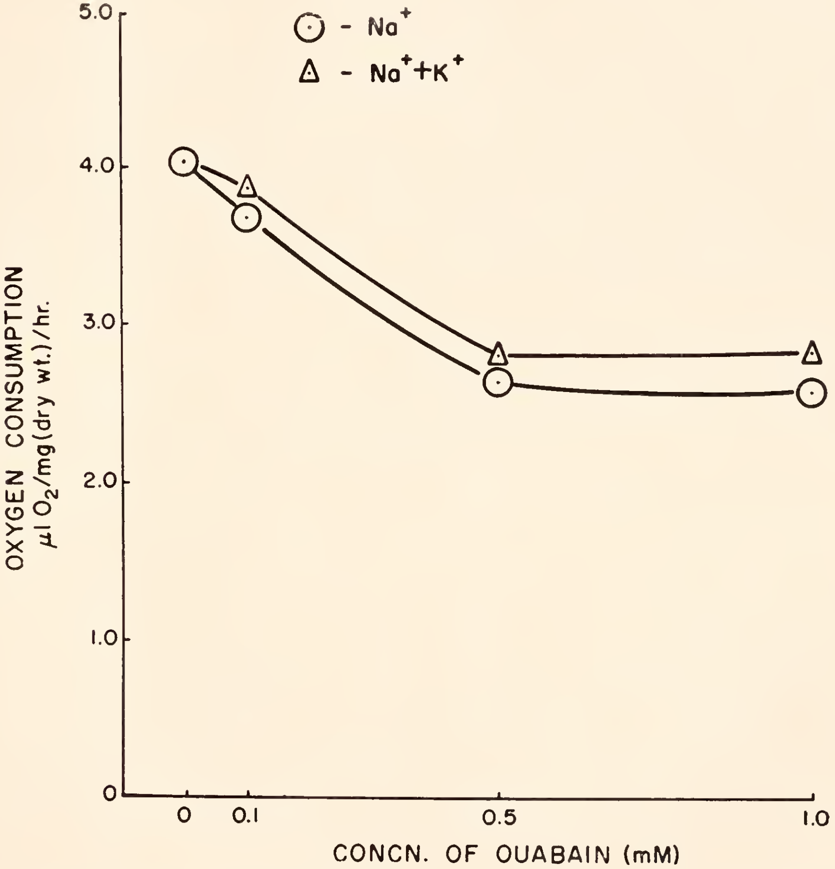


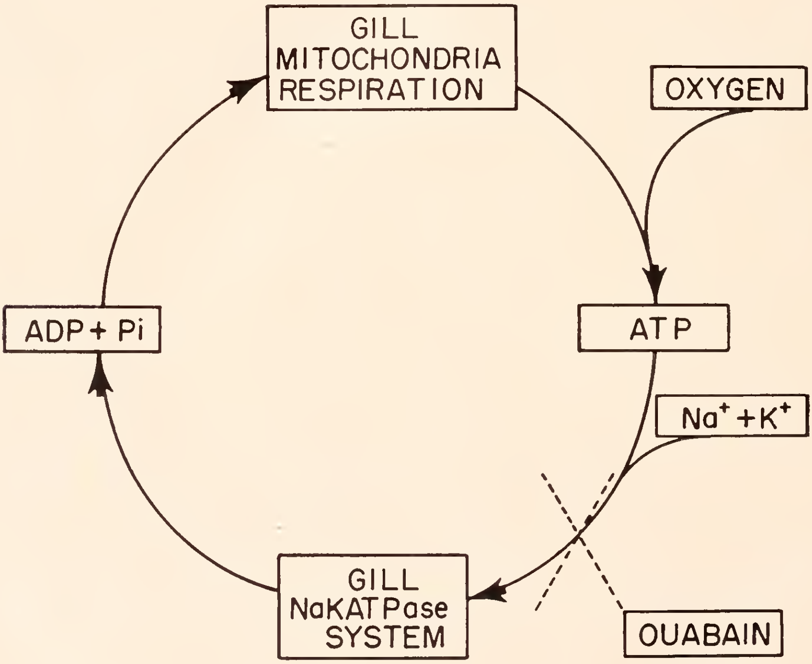
FIGURE 6. Effect of ouabain on Na^+- and $Na^+ + K^+$-stimulated respiration in the excised gill. Individual crabs were maintained in 2 l. of tap H_2O for 2 weeks prior to the experiments. The Qo_2 was measured in 50 m*M*/l. Tris containing 310 m*M*/l. Na^+, or 310 m*M*/l. $Na^+ + 6.6$ m*M*/l. K^+ (final concentrations). The final ouabain concentrations are shown on the graph. Each point is an average of up to 9 different crabs.

Varying K^+ concentration from 0 to 10.2 m*M*/l. caused no significant alteration in excised gill Q_{O_2} (Fig. 4). Choline chloride in the concentration range 0–10.2 m*M*/l. likewise caused no change in oxygen consumption (Fig. 4). There was no indication of a synergistic increase in oxygen consumption (Fig. 5) when both Na^+ and K^+ ions were added to the medium; indeed, the oxygen consumption was reduced from that observed when Na^+ alone was added (Fig. 3). Choline chloride at equivalent osmolar concentrations caused no apparent change in gill Q_{O_2} (Fig. 5).

Na^+ and K^+ are both required for maximal NaKATPase activity. The lower level of oxygen consumption observed when both ions were added, as opposed to the Q_{O_2} in the presence of Na^+ alone, suggests that K^+ has a different role in tissue respiration than in the NaKATPase system.

Since ouabain inhibits the oxygen consumption of excised gill tissue measured in 75% sea water, the inhibitory effect of ouabain was tested on Na^+-, and $Na^+ + K^+$-stimulated oxygen consumption (Fig. 6). Low concentrations of ouabain reduced both the Na^+-, and the $Na^+ + K^+$-stimulated gill Q_{O_2}.

Assuming that the crustacean gill participates in ionic regulation and that gill NaKATPase activity is a basic mechanism of membrane transport, it is concluded that a portion of the energy derived from mitochondrial respiration supports ionic regulation. The following model (Fig. 7) summarizes the various steps that are proposed to occur in crustacean ionic regulation:



Energy, in the form of ATP, is derived from gill mitochondrial respiration. Some of this energy is utilized by the NaKATPase system to drive Na^+ and K^+ membrane transport. The hydrolysis of ATP by NaKATPase results in the production of ADP and Pi which support gill mitochondrial respiration. It is suggested that ouabain inhibits gill Na^+- and $Na^+ + K^+$-stimulated oxygen consumption by inhibiting NaKATPase activity, decreasing the amounts of ADP and Pi available to the mitochondria, thus reducing total oxygen uptake. These observations are consistent with the hypothesis that some of the energy derived from respiration in the gill is employed in ion regulation.

The authors wish to thank Miss Sheryl Corcoran for skillful technical assistance.

Summary

1. The Q_{O_2} of excised *Cardisoma guanhumi* gill tissue is higher in hypo-osmotic than in hyperosmotic sea water.

2. Gill tissue Q_{O_2} is maximally stimulated by Na^+; K^+ does not stimulate Q_{O_2}; addition of Na^+ and K^+ simultaneously causes no greater stimulation of oxygen uptake than that following Na^+ alone.

3. Ouabain, a Na^+ transport inhibitor, reduces Na^+-, and $Na^+ + K^+$-stimulated gill tissue respiration. In addition, Q_{O_2} of gill tissue measured in 75% sea water is reduced by ouabain.

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PHYSICAL AND CHEMICAL COMPARISONS OF UNIVALENT AND MULTIVALENT SEA URCHIN FERTILIZINS[1]

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Solutions of most sea urchin egg jellies can agglutinate the sperm of the species (Lillie, 1913; Tyler, 1940; Vasseur, 1951; for most recent review see Monroy, 1965). This reaction is usually species-specific (Tyler, 1949) and is believed to be analogous to antibody agglutination of cells. The sperm agglutinin, called fertilizin, is the principal component of the egg jelly (Tyler, 1941).

In keeping with immunological doctrine, Tyler (1948) proposed that agglutinating fertilizin must be multivalent in terms of the combining groups (active sites) on the molecule. Tyler (1941, 1942, 1948) and Metz (1942, 1954) showed that fertilizin could be rendered non-agglutinating (univalent) without destroying its capacity to combine with the specific complementary sites of the sperm.

The chemistry of various multivalent sea urchin fertilizins has been studied. Tyler and Fox (1940) obtained a positive biuret test with *Strongylocentrotus purpuratus* fertilizin. This and the fact that proteolytic enzymes destroyed the sperm-agglutinating properties of their preparations, was interpreted to mean that the fertilizin was of a protein nature. Vasseur (1948) showed that the fertilizins of *Paracentrotus lividus, Echinus esculentus, Stronglyocentrotus droebachiensis,* and *Echinocardium cordatum* contained nine different amino acids. The nitrogen content of these different fertilizins varied from 2% to 5% of the dry weight. Sulfur and sulfate analyses indicated that the materials contained roughly 25% to 30% sulfate. Vasseur (1952), in summary of these and other findings, concluded that fertilizins in general are 20% to 25% protein and 75% to 80% carbohydrate, with sulfate groups esterified to the carbohydrate. Monroy (1965) reviews the chemical compositions of several fertilizins.

Sulfates esterified to carbohydrates would tend to give the fertilizin molecule a negative charge at neutral pH. The negative charge of the molecule was confirmed by Runnström *et al.* (1942) and Tyler (1949, 1956) who demonstrated that fertilizin migrates during Tiselius electrophoresis as a single component with high anodic velocity. The mobility changes very little over the pH range of 3 to 9.

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[3] It is with great pleasure that the author acknowledges the support and guidance of Dr. C. B. Metz. The author is indebted to Mr. C. R. Windsor for teaching him the operation of the amino acid analyzer.

The sedimentation behavior (Runnström *et al.,* 1942; Tyler, 1956) shows that the molecule is a high molecular weight, asymmetric particle. The sedimentation velocity increases with decreasing concentration for *Psammechinus miliaris* (Runnström *et al.,* 1942) and *Arbacia punctulata* (Tyler, 1956) fertilizins.

The relationship of univalent to the multivalent fertilizins has not been studied seriously by chemical and physical techniques. Tyler (1941), and Metz (1957) hypothesized that the conversion to the univalent form involves a fragmentation of the fertilizin molecule. Fragments containing the active sites for combination with sperm must be relatively large since they fail to pass through dialysis membranes (Tyler, 1941; also see Krauss, 1949). The only direct evidence for fragmentation is found in experiments of Hathaway and Metz (1961) who demonstrated release of an inert fertilizin component(s) following spontaneous reversal of agglutination. These workers, however, did not establish whether fragmentation is a uniform process resulting in one or several subunits, comparable to antibody fragmentation to a univalent form (Porter, 1959); or whether cleavage of the molecule is random, involving formation of a spectrum of fragments; or whether it simply involves a progressive destruction of active sites leaving only one in the final univalent material (Tyler, 1941).

The present work describes some of the physical and chemical properties of univalent *Lytechinus variegatus* fertilizin and includes comparison with those of the multivalent form.

Materials and Methods

1. *Preparation of fertilizin*

Lytechinus variegatus was collected at Alligator Harbor, Franklin County; Biscayne Bay, Dade County; and Key Largo, Monroe County, Florida. Gametes were obtained either by injecting the animals with isotonic KCl or by cutting the test, which caused shedding of the gametes. Semen was collected without added sea water in Syracuse dishes. Eggs were collected in Marine Biological Laboratory artificial sea water (Formulae and Methods, IV, 1956) and washed three times with this sea water. To prepare egg jelly solutions, the washed eggs were resuspended in artificial sea water, pH 4.5 to 4.9, to a final concentration of approximately 20% by volume. Only those samples which were free from cytolyzed eggs upon microscopic examination were used for further study. After the eggs settled, the supernatant was decanted and centrifuged in a hand-driven centrifuge. The pH was adjusted to 7.5 with NaOH or $NaHCO_3$, and the supernatant solution was centrifuged at 25,000 *g* for 30 minutes at 4° C. to remove any particles. Egg jelly (fertilizin) was then precipitated with a final concentration of 65% ethanol below 0° C. The precipitate was suspended in distilled water, and the suspension dialyzed in the cold against several changes of distilled water, for a maximum time of 72 hours. The undissolved material was removed by centrifuging at 30,000 *g* for 30 minutes at 4° C. See Figure 1 for flow sheet of preparation. The preparations were stored either frozen or at 4° C. Fertilizin retained its sperm-agglutinating capacity for at least one month at 4° C. and at least one year when frozen.

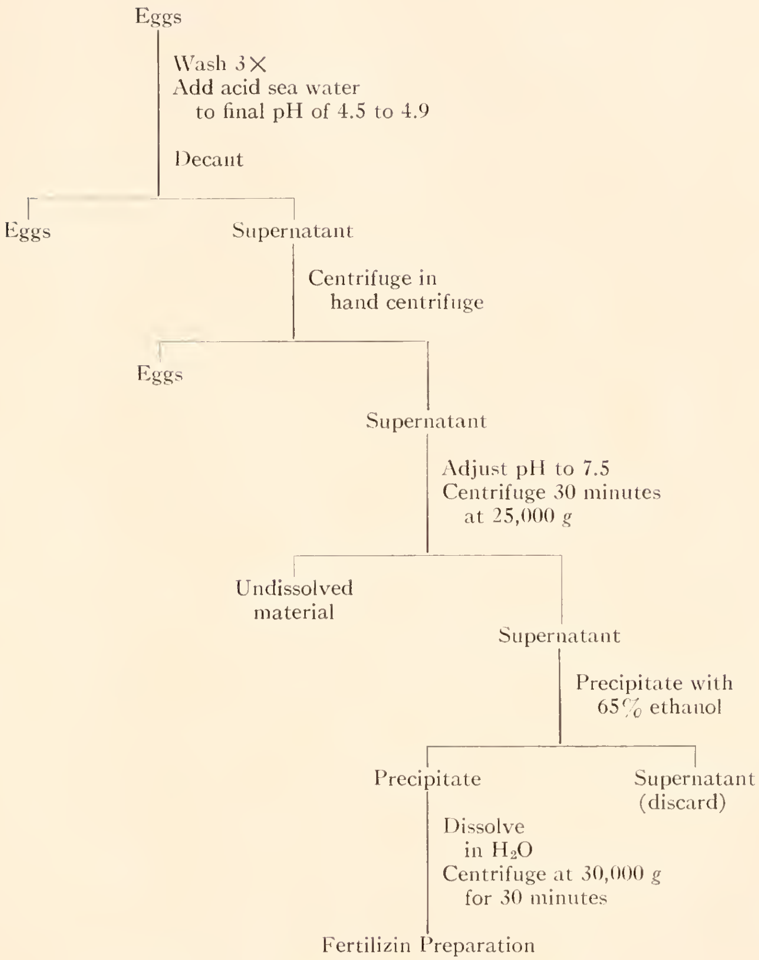
Eggs
Wash 3×
Add acid sea water
to final pH of 4.5 to 4.9
Decant
Eggs
Supernatant
Centrifuge in
hand centrifuge
Eggs
Supernatant
Adjust pH to 7.5
Centrifuge 30 minutes
at 25,000 g
Undissolved
material
Supernatant
Precipitate with
65% ethanol
Precipitate
Supernatant
(discard)
Dissolve
in H₂O
Centrifuge at 30,000 g
for 30 minutes
Fertilizin Preparation

FIGURE 1. Isolation procedure of fertilizin.

2. *Preparation of univalent fertilizin*

Fertilizin was converted to the univalent form by a 30-minute treatment with 2% hydrogen peroxide at room temperature (Metz, 1954), followed by dialysis against distilled water or other solvent. Control multivalent fertilizin solutions were treated identically except that water was substituted for hydrogen peroxide.

Operationally multivalent fertilizin samples were defined as those which could agglutinate homologous sperm. A sample was considered univalent when it failed to agglutinate homologous sperm, and when, in addition, these treated sperm could not subsequently be agglutinated by a sample of multivalent fertilizin. Agglutination tests were made by mixing equal volumes of 1% sperm suspensions and the test sample in a Syracuse dish. A control of sperm mixed with sea water was always included.

Most comparisons were made between univalent fertilizin formed by hydrogen peroxide treatment and the parent multivalent material. Univalent fertilizin was also obtained by two other techniques: (1) a spontaneous breakdown of multivalent fertilizin, at times enhanced by an elevation of temperature, and (2) treatment with proteolytic enzymes: (trypsin [Worthington, crystallized], chymotrypsin [C. F. Boehring and Soehne], and pronase [Cal. Biochem., B grade]). Trypsin and chymotrypsin were buffered with 1*M* pyridine: collidine buffer, pH 8.0. Pronase was buffered with 1 *M* pyridine: collidine buffer, pH 8.0, containing 0.02 *M* $CaCl_2$. This volatile buffer was used to avoid salt effect which could interfere with electrophoretic and chromatographic analyses of the hydrolysates.

3. *Physical analyses*

a. Viscosity. Viscosities were determined in a Cannon-Ubbelohde semi-micro dilution viscometer, and an Ostwald-Cannon-Fenske number 200 viscometer with temperature controlled to ±0.1° C.

b. Sedimentation velocities. Sedimentation velocity values were determined in a Spinco Model E ultracentrifuge at a speed of 47,660 rpm. Usually two samples were run at a time, using one normal and one wedge cell. The solvent was 0.1 *M* NaCl. Schlieren optics were employed to detect the position of the boundary. Peak displacement was measured on a Nikon Profile Projector at a magnification of 10 × or 20 ×. Sedimentation values were calculated from the slope of the line obtained by plotting 2.3 log x against time (Schachman, 1959). All values were corrected to standard conditions of water as a solvent at 20° C. by the approximation

$$s_{20,w} = s \frac{\eta_t}{\eta_{H_2O}} \text{ (Steiner, 1965).}$$

Solvent viscosity values were obtained from the International Critical Tables (1926) and Lange (1956).

c. Electrophoresis. Electrophoresis on cellulose acetate (Sepraphore III, Gelman Instrument Company) was carried out utilizing barbital buffer, pH 8.6; borate buffer, pH 8.0 and 8.6; and phosphate buffer, pH 6.5, 7.5, and 8.0, $\Gamma/2 = 0.05$. Metachromatic staining in all procedures was with 0.01% Toluidine Blue O in distilled water. Protein staining was attempted with Amido Schwarz or Ponceau S. Samples were destained with 2% acetic acid or 0.001 N HCl. Photography of the stained cellulose acetate strips was performed by making contact negatives of the strips on Kodak Contrast Process ortho film.

4. *Chemical analyses*

a. Carbohydrate analyses. Material for carbohydrate analysis was hydrolyzed in sealed tubes with 1 N HCl at 100 ± 2° C. for from 1 to 24 hours. Excess acid was removed by drying *in vacuo* in the presence of NaOH and H_2SO_4 or P_2O_5. Subsequently, the material was dissolved in distilled water and dried again. After the second drying, the hydrolysate was dissolved in a minimal amount of water and aliquots were applied to Whatman number 1 or 3 MM filter papers for ascending chromatography. Solvent systems used were 1-butanol:ethanol:acetone:water, 5:4:3:2 (Gray and Fraenkel, 1954) and 1-butanol:pyridine:water, 54:25:45 (Block *et al.*, 1958). Spots were developed with ammoniacal silver nitrate or aniline-phosphoric acid (Block *et al.*, 1958). Chromatographic comparisons were made with arabinose, glucose, galactose, fructose, rhamnose, fucose, glucosamine, and galactosamine (all Nutritional Biochemical Corporation) standards. Rhamnose and fucose from Cal. Biochem. were also used. Fucose determinations were done by the CyR3 method of Dische and Shettles (1948). Optical densities were determined in a Beckman DK II spectrophotometer, a Bausch and Lomb Spectronic 20, or a Zeiss PM Q II spectrophotometer.

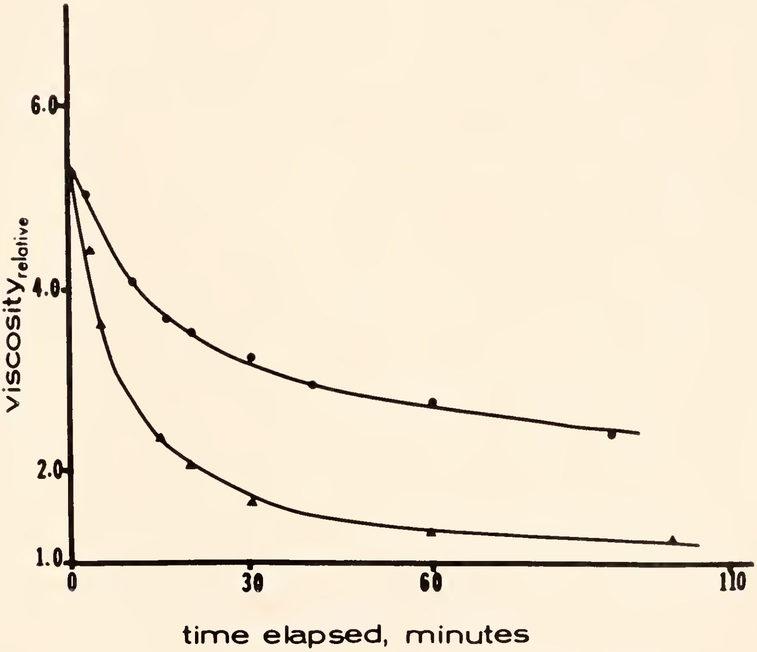

FIGURE 2. Viscosity change of fertilizin in two concentrations of H_2O_2. Fertilizin concentration 0.6 mg./ml. Upper curve: 0.4% H_2O_2. Lower curve: 2.0% H_2O_2. The viscosity of a 5-ml. sample was determined in two Ostwald-Cannon-Fenske viscometers at 30° C. Thirty per cent H_2O_2 was added to one viscometer to attain a 2% or 0.4% concentration. An equivalent amount of water was introduced into the other viscometer. The viscosities of the experimental and control solutions were determined at intervals. No change was noted in the control solution in 100 minutes.

b. Amino acid analyses. Amino acid contents of univalent and multivalent fertilizins were determined with a Phoenix Model K-5000 automatic amino acid analyzer employing Beckman or Phoenix resins. For the analysis, material was hydrolyzed, with 6 N HCl in sealed evacuated tubes at 110 ± 2° C. for 24 hours or by treatment with the enzyme pronase.

c. Nitrogen. Nitrogen was determined by the Kjeldahl procedure (Fischer, 1961).

TABLE I

Sedimentation velocities of multivalent and univalent Lytechinus fertilizins

Sample	Concentration g. fucose/ml.		$s_{20,w} \times 10^{13}$	
	m	u	m	u
8/31, 4	195	750*	6.15	2.78
8/31, 3	175	700*	5.83	2.87
5/12, 2	260	—	5.76	—
5/12, 1	300	—	5.05	—
9/1	400	1025*	4.48	2.78
9/4, 1	750*	—	3.18	—
10/1	—	220	—	2.96

* Conc. with Aquacide.
m: Multivalent fertilizin.
u: Univalent fertilizin.

d. Amino sugars. Glucosamine and galactosamine were determined on the Phoenix amino acid analyzer employing Beckman resins. Samples were hydrolyzed with 2 N HCl for 1, 2, 5, and 10 hours in sealed tubes at 105 ± 2° C. Dry weights of samples were determined on a Mettler balance of material dried by lyophilization. Several samples of multivalent and univalent *Lytechinus* fertilizins were concentrated by dialysis against Aquacide (Cal. Biochem.).

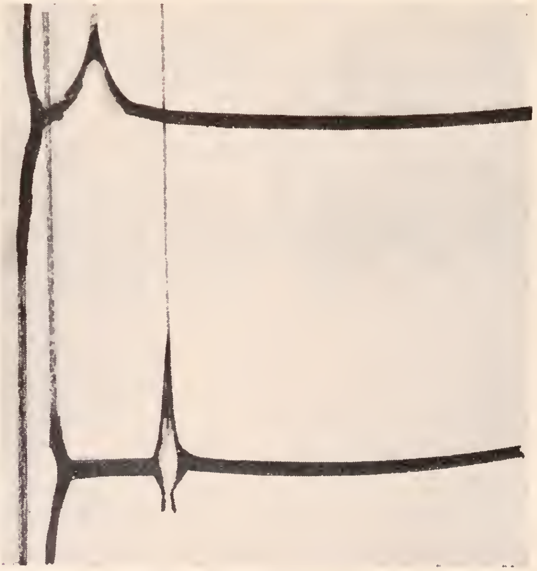

FIGURE 3. Sedimentation pattern of multivalent and hydrogen peroxide formed univalent fertilizins. Speed was 47,660 rpm at 10° C. Time illustrated is 64 minutes after speed was attained. Bar angle of 45°. Upper pattern: Sample 8/31, 4 U. Lower pattern: Sample 8/31, 4 M.

RESULTS

Viscosity

Viscosities of four univalent and parent multivalent preparations were compared. In all cases the conversion (H_2O_2 treatment) to the univalent form resulted in a marked drop in relative viscosity.

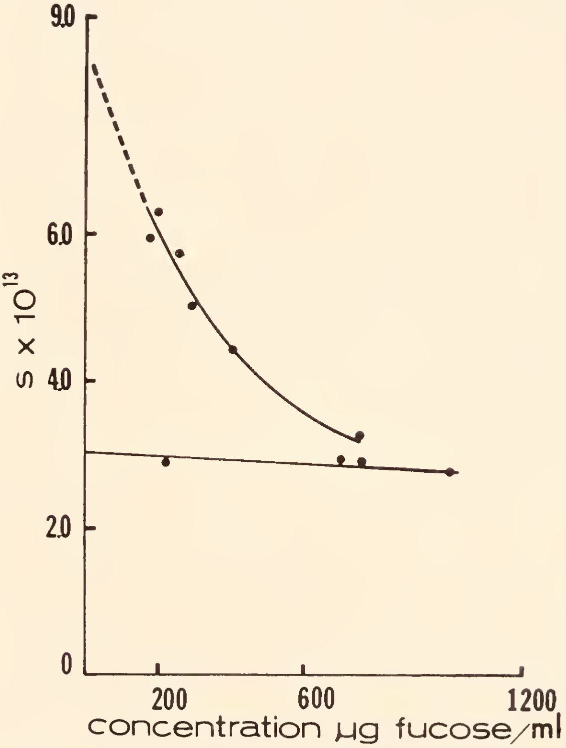

FIGURE 4a. Sedimentation velocity *vs.* concentration for multivalent and hydrogen peroxide formed univalent fertilizin. Upper curve: Multivalent fertilizin. Lower curve: Univalent fertilizin.

The viscosity change was followed with three different samples of fertilizin. Data from one of these are given in Figure 2. The rate of change is seen to be dependent on the H_2O_2 concentration. Following the completion of the experiments the samples were dialyzed against 0.1 *M* NaCl for 10 hours at 4° C. After dialysis the treated samples were found to be electrophoretically indistinguishable from univalent fertilizin formed by 2% H_2O_2 treatment for 30 minutes.

Similar experiments were conducted with fertilizins of *Tripneustes esculentus, Echinometra lucunter, Eucidaris tribuloides,* and *A. punctulata.* In all cases a drop in relative viscosity occurred in the presence of hydrogen peroxide, whereas the viscosity of control preparations containing no hydrogen peroxide showed no viscosity change even after 100 minutes at 30° C.

Sedimentation velocity determinations

Concentrations and $s_{20,w}$ values for multivalent and hydrogen peroxide formed univalent fertilizins are given in Table I. Photographs were taken at 16-minute intervals, the first exposure being made when speed was attained. A typical sedimentation pattern is illustrated in Figure 3.

The variation of the sedimentation coefficient of multivalent fertilizin with decreasing concentration (Fig. 4a, b) shows the behavior expected for a highly asymmetric molecule; the univalent material behaves as a more symmetric system.

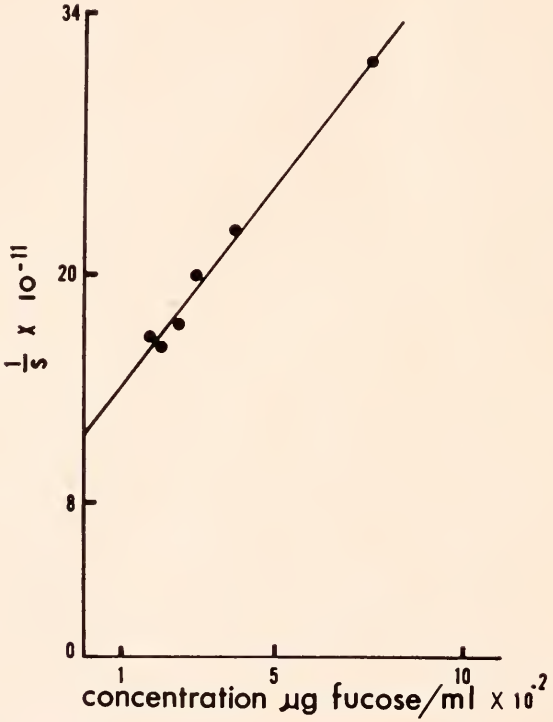

FIGURE 4b. 1/s *vs.* concentration for multivalent fertilizin.

With decreasing concentration the univalent peak tends to spread, suggesting heterogeneity (Tanford, 1961). The empirical sedimentation velocity behavior of asymmetric particles with concentration is given by the formula

$$1/s = 1/s^{\circ} + kc \text{ (Tanford, 1961).}$$

A plot of 1/s *vs.* c is a more reliable method of obtaining s° for multivalent fertilizin than the plot of s *vs.* c (Fig. 4b). Extrapolation to infinite dilution gives a value of 8.60 Svedberg for multivalent *Lytechinus* fertilizin and 3.05 Svedberg for univalent fertilizin formed by hydrogen peroxide treatment (Fig. 4a).

Electrophoresis

Freshly prepared multivalent fertilizin failed to migrate from the origin under the electrophoretic conditions employed. Univalent fertilizin formed by treatment with hydrogen peroxide is resolved into four bands, as determined by metachromatic staining (at least 50 trials). When multivalent fertilizin is retained at room temperature for several hours a spontaneous breakdown of the material is noted from its electrophoretic behavior. With prolonged storage at 37° C., *e.g.* 150 hours, no material remains at the origin on the cellulose acetate strips and the material is functionally totally univalent. This univalent material formed by spontaneous breakdown of the multivalent form produces a diffuse streak rather than discrete bands in cellulose acetate electrophoresis, indicating that it is more heterogeneous than the univalent material formed by hydrogen peroxide. The rate of migration of the fastest moving fraction never exceeds that of the fastest moving component of the hydrogen peroxide formed univalent material. The spontaneous breakdown of the fertilizin may be due to the presence of digestive enzymes which were not removed during the washings of the eggs. Figure 5 shows the typical behavior of the different types of univalent fertilizin compared to the multivalent form.

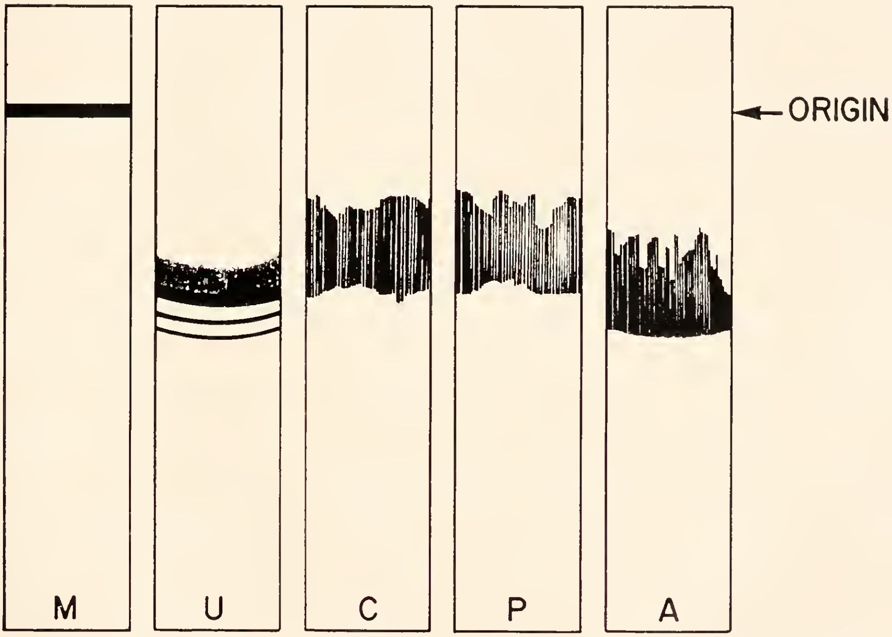

FIGURE 5. Cellulose acetate electrophoretic patterns of *Lytechinus* fertilizins. The solvent was borate buffer, pH 8.6 $\Gamma/2 = 0.05$. Two hundred volts were applied for 25 minutes. M: Multivalent fertilizin. U: Univalent fertilizin formed with hydrogen peroxide. C: Univalent fertilizin formed by treatment with chymotrypsin for 30 minutes at 30° C. P: Univalent fertilizin formed by treatment with pronase for 30 minutes at 30° C. A: Autodegraded univalent fertilizin formed by storing multivalent fertilizin at 37° for 50 hours.

The effects of the proteolytic enzymes, chymotrypsin and pronase, on the viscosity, electrophoretic behavior, and sperm-agglutinating properties were studied with three different samples of multivalent fertilizin. The addition of trypsin to a solution of multivalent fertilizin resulted in a precipitation. The addition of 300 μg. of pronase to a 5 ml. solution of multivalent fertilizin of relative viscosity 3.73 resulted in a decrease of the viscosity to 1.94 within one minute after the addition of the enzyme. At the end of that time the material was also univalent, as determined by sperm agglutination tests. Chymotrypsin-treated material was univalent on the basis of sperm agglutination 30 minutes after the addition of the enzyme. Figure 5 gives the electrophoretic patterns obtained with samples of fertilizin rendered univalent by treatment witht chymotrypsin and with pronase for 30 minutes. The electrophoretic behavior of the treated samples suggests that the enzymes acted on the original material in a degradative manner.

It seemed of interest to determine the effect of H_2O_2 on "spontaneously" formed univalent fertilizin since this produces a broad streak rather than discrete bands in electrophoresis. Two samples of spontaneously formed univalent fertilizin were treated with 2% hydrogen peroxide. The samples were univalent in terms of sperm agglutination and contained only migrating electrophoretic material. Two samples that were multivalent in terms of sperm agglutination, but which did contain some migrating material (partially degraded), were also treated. In all four cases the resultant univalent material produced the four metachromatic bands characteristic of the H_2O_2 formed univalent material.

These observations, and the course of viscosity change, suggest that intermediate forms may occur during hydrogen peroxide degradation of fertilizin. It was further suggested that these intermediates may be very similar in electrophoretic behavior to spontaneously formed univalent fertilizin.

The demonstration of such an intermediate(s) was carried out by arresting the action of H_2O_2 at intervals by addition of catalase to the fertilizin-hydrogen peroxide mixture. Two samples of multivalent fertilizin were treated with 2% hydrogen peroxide. At intervals, 4-ml. aliquots were removed and 5 μg. (5 λ) of the enzyme were added to the aliquot. A rapid evolution of gas, presumably oxygen, occurred when the catalase was added. Gas evolution lasted for several minutes. The last aliquot was removed at 120 minutes. At the end of that time 5-λ samples of each aliquot were applied to cellulose acetate strips and electrophoresis was carried out in barbital buffer, pH 8.6. Alteration in electrophoretic behavior was rapid.

At the end of 10 minutes of treatment no material could be observed at the origin. The migrating material behaved as predicted. A broad migrating band was observed following a 1-minute treatment with H_2O_2. At the end of 20 minutes of treatment four metachromatic bands were observed. No change was noted over the next 100 minutes of reaction time.

One of the two samples was also treated with a lower concentration of hydrogen peroxide. Five milliliters of sample was treated with 0.6% hydrogen peroxide. Aliquots of 0.5 ml. were removed at intervals of 0, 5, 10, 20, 30, 60, and 135 minutes after the addition of the peroxide, and 1 μg. of catalase was added. As expected the breakdown of the material was much slower in this case. At the end of the 30 minutes some non-migrating material was still demonstrable; at 60 minutes all material migrated; but breakdown identical to the normal univalent pattern was not noted until 135 minutes.

Amino acid analysis

Four samples each of multivalent and the hydrogen peroxide formed univalent fertilizins were examined for amino acid composition. At least 16 amino acids were found in each of the hydrolysates. In all runs two peaks appeared on the 50-cm. column after phenylalanine, and before lysine on the 10-cm. column. These peaks have been tentatively identified as glucosamine and galactosamine on the basis of their R_f values compared to published values (Walborg *et al.,* 1963) and to standard amino acid-hexosamine (as hydrochlorides) mixtures. The R_f values of the two peaks are identical with the R_f values of the standard sugars on both the 50-cm. and 10-cm. columns. Quantitative calculations were based on values obtained for standards on the 10-cm. column. A high degree of similarity was noted between the amino acid compositions of the univalent and parent multivalent form. There were, however, differences in some amino acids. These differences were not uniform in all of the analyses. Further analyses are required in order to make a definitive statement concerning the amino acid compositions of multivalent and univalent fertilizins.

Free amino acids were found in all four multivalent fertilizin samples hydrolyzed with pronase.

Protein staining

Protein staining was attempted with Amido Schwarz and Ponceau S on cellulose acetate strips. No detectable protein-staining material was found in preparations of either multivalent or hydrogen peroxide formed univalent fertilizins. Protein tests on the cellulose acetate strips were done with different multivalent fertilizin solutions varying in concentration from 0.05 mg./ml. to 2.0 mg./ml., and with univalent fertilizin solutions over the concentration range of 0.05 mg./ml. to 3.0 mg./ml. Ten λ of each solution were applied to the strips.

Nitrogen

Samples for nitrogen determination were dried by lyophilization and weighed. Kjeldahl determinations were then made on the dried material. Determinations on eight different samples of multivalent fertilizin yielded a value of 2.37% nitrogen, with a range of 2.12% to 2.87%.

Amino sugars

A total of three univalent and eight multivalent samples of known weight were hydrolyzed under conditions generally employed for amino sugars. The data are given in Table II. Maximal release of glucosamine and galactosamine seems to occur between 5 and 10 hours. The total amount of amino sugars found is of the order of 1% of the dry weight of the fertilizin.

One sample each of *Tripneustes* and *Echinometra* fertilizins was also found to contain peaks corresponding to the amino sugar peaks.

Table II

Amino sugar content of Lytechinus fertilizins. Gram Residues per 100 grams material

Sample	Hours of hydrolysis	Glucosamine	Galactosamine
11/16 M	1	0.22	0.15
1/24 M	1	0.19	0.15
11/24 M	2	0.32	0.27
11/24 M	2	0.33	0.35
2/7 M	2	0.31	0.23
2/7 U	2	0.38	0.23
9/1 M	5	0.68	0.37
9/1 M	10	0.46	0.25
11/16 M	10	0.80	0.34
11/24 U	10	0.53	0.36
11/18 U	10	0.59	0.31

Carbohydrate analysis

Fucose was the only carbohydrate found by chromatography in hydrolysates of *Lytechinus* fertilizin. No non-methyl pentose was detected by the method of Dische and Shettles (1948).

Table III

Fucose analyses of Lytechinus fertilizins

A. Comparison of samples after dialysis

Sample	Concentration μg. fucose/ml.	
	Multivalent	Univalent
6/30/64	23.9	25.8
6/18/64	34.4	30.6
5/12/65	190.0	189.0
5/16/65	218.0	224.0
5/17/65	180.0	149.0
6/8/65	171.0	180.0

B. Fucose content of samples of known weight

Sample	% Fucose	
	Multivalent	Univalent
6/13/64	26.8	27.2
10/1/65 (1)	26.9	20.5
10/2/65	24.3	23.3
10/1/65 (2)	22.8	22.8
10/8/65	30.4	33.8
10/7/65	28.9	24.6
Average	26.7	25.5

Comparison of the fucose content of multivalent fertilizin and univalent fertilizin formed by hydrogen peroxide treatment was made on dried samples and samples obtained immediately after dialysis. Data comparing the fucose content of univalent and multivalent fertilizins are given in Table III. The fucose content of the two materials is identical.

Discussion

These experiments demonstrate that the formation of univalent fertilizin is associated with a breakdown of the multivalent form. The different methods of preparing univalent fertilizin yield products which are physiologically equal in terms of action on sperm, but are different in physical behavior.

The existence of different physical forms of univalent fertilizin has been demonstrated by comparison of the electrophoretic behavior of univalent fertilizins formed by the action of hydrogen peroxide, proteolytic enzymes, and autodegradation. Of the three univalent forms studied, the most completely degraded, in terms of relative viscosity and electrophoretic behavior, is the one formed by the action of hydrogen peroxide. The observation that autodegraded univalent fertilizin can be further degraded to a form which is electrophoretically equivalent to the univalent fertilizin formed with hydrogen peroxide, suggests that the linkages cleaved during autodegradation may be the same or very similar to those initially attacked by hydrogen peroxide. This is further substantiated by the fact that during the hydrogen peroxide degradation of the multivalent fertilizin, it is also possible to demonstrate the existence of a form similar to the autodegraded form.

The formation of a univalent fertilizin by treatment with hydrogen peroxide has been shown to be dependent at least on the concentration of the peroxide (Fig. 2), for *Lytechinus* fertilizin. The degradation reaction does not proceed instantaneously for any of the five species tested. Hydrogen peroxide is known to degrade polysaccharides (see Moody, 1964 for review) but the mode of action is not clear. Metz (1942) obtained univalent fertilizins from *A. punctulata* and *S. purpuratus* by subjecting multivalent fertilizins to ionizing radiation. It is not clear whether the formation of univalent fertilizins in this case was due directly to the radiation or a secondary formation of peroxides from water (Moody, 1964).

The $s^\circ_{20,w}$ values of 8.60 and 3.05 obtained for multivalent, and hydrogen peroxide formed univalent, *Lytechinus* fertilizins, respectively, demonstrate that depolymerization has occurred in transition from the multivalent to the univalent form. Krauss (1949) suggested that univalent fertilizin is a depolymerization of multivalent fertilizin on the basis of the mucin clot reaction. Tyler (1949, 1956) and Runnström *et al.* (1942) obtained sedimentation velocity values for various multivalent sea urchin fertilizins. Unfortunately neither worker obtained an infinite dilution sedimentation constant. They were, however, able to conclude that the multivalent fertilizins of *P. miliaris* and *A. punctulata* behave as asymmetric particles in the ultracentrifuge. In the present work this was also found for the multivalent fertilizin of *L. variegatus.*

The chemical analyses reported here were done mainly to compare multivalent fertilizin and univalent fertilizin formed by hydrogen peroxide treatment. The glucosamine and galactosamine found in *Lytechinus* fertilizin are the first clear demonstration of such amino sugars in any fertilizin. Previous determinations of amino sugars as components of fertilizins (see Vasseur, 1952) were not clear because of the interference of amino acids and carbohydrates in the test procedure.

The experiments of Hathaway and Metz (1961) indirectly suggest that univalent fertilizin may be composed of several fractions, not all of which combine with sperm. The electrophoretic behavior of univalent *Lytechinus* fertilizin formed with hydrogen peroxide shows that the material is composed of at least four metachromatic fractions. Stern and Metz (unpublished data) have been able to remove some of these fractions with homologous sperm. Since in no case were all of the fractions removed it is suggested that some portion(s) of the parent multivalent fertilizin are inactive in terms of sperm agglutination.

The structural relationship of the univalent fertilizin fractions to the parent multivalent form is at present not known. A chromatographic investigation similar to that conducted by Porter (1959, 1960) on rabbit antibodies might prove of interest in interpreting the structure of multivalent fertilizin.

SUMMARY

1. Treatment of multivalent *Lytechinus* fertilizins with hydrogen peroxide, proteolytic enzymes, or elevated temperatures results in the formation of univalent fertilizin.

2. Electrophoretic behavior showed that univalent fertilizins formed by the different means listed above are not physico-chemically equivalent. During the course of hydrogen peroxide action, forms intermediate to the initial material and final product are realized. Hydrogen peroxide formed univalent material is composed of at least four fractions as demonstrated by metachromatic staining on cellulose acetate. This suggests that degradation of fertilizin by hydrogen peroxide is nonrandom.

3. The main carbohydrate found in hydrolysates of *Lytechinus* fertilizins was the methyl pentose, fucose. The fucose content of multivalent and hydrogen peroxide formed univalent fertilizins was found to be identical following dialysis. Glucosamine and galactosamine were found to be components of *Lytechinus* fertilizin.

4. On the basis of sedimentation velocities, electrophoretic behavior, and lowered relative viscosity it is concluded that univalent fertilizin is a fragmentation product of multivalent fertilizin.

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AUTHORS' ERRATUM

Pikó, L., A. Tyler, and J. Vinograd: In the paper "Amount, location, priming capacity, circularity and other properties of cytoplasmic DNA in sea urchin eggs" published in BIOLOGICAL BULLETIN, Vol. 132, No. 1, the following correction should be made on Page 71, line 14 from top: change "mg." to μg.".

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HEMOLYMPH PROTEINS AND REPRODUCTION IN PERIPLANETA AMERICANA: THE NATURE OF CONJUGATED PROTEINS AND THE EFFECT OF CARDIAC–ALLATECTOMY ON PROTEIN METABOLISM[1]

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That cardiacectomy results in a loss of urates from the fat bodies (Bodenstein, 1953) of the American cockroach is indicative of a role for the corpora cardiaca in protein metabolism, and it is quite possible that this effect may be related to the neurosecretory factors abundantly stored in this organ (Scharrer, 1955). The corpora cardiaca are also known to play a role in carbohydrate metabolism: administration of semi-purified extracts of the cardiaca results in this species in increased blood trehalose levels at the expense of fat body glycogen, and this has been found to be the result of increased phosphorylase activity (Steele, 1961, 1963). Evidence has accumulated to suggest that the corpus allatum plays a role in lipid and protein metabolism in female *Periplaneta americana:* allatectomy induces fat body hypertrophy (Bodenstein, 1953; Mills, Greenslade and Couch, 1966) and slows the turnover of phospholipid and triglyceride fractions (Vroman, Kaplanis and Robbins, 1965); it affects RNA synthesis (Thomas and Nation, 1966b) and results in adult females in increased blood protein concentration due to non-utilization (Mills, Greenslade and Couch, 1966). The corpora allata are known particularly to influence the formation and utilization of a sex-specific protein in adult females of *Periplaneta americana* (Menon, 1963, 1965; Adiyodi and Nayar, 1966; Thomas and Nation, 1966a). The nature of the conjugated proteins of this species in relation to the molting and clotting processes has been described in some detail by Siakotos (1960a, 1960b), using paper electrophoresis and various staining procedures. Very little information, however, is available regarding the behavior of the conjugated proteins in relation to reproduction and the effect of the total removal of the cardiacum-allatum complex on the biosynthesis, chemical composition and mobilization of plasma protein fractions in adult females of *Periplaneta.*

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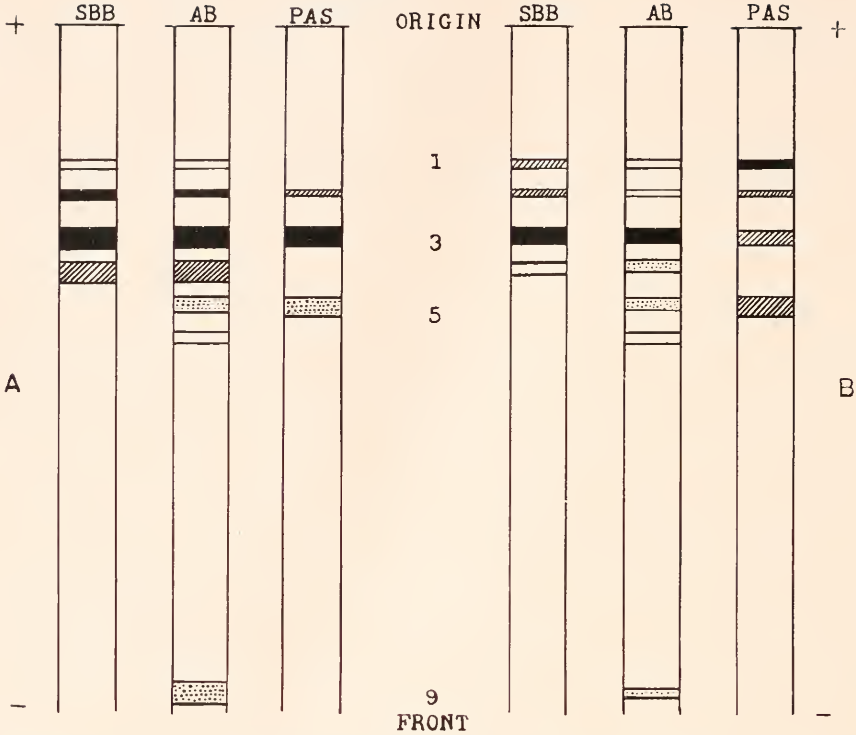

FIGURE 1. Electrophoretogram showing the nature of hemolymph proteins during the preovipositional period in adult female *Periplaneta americana*. Length of basal oocytes (A) 2.70 mm. and (B) 3.36 mm. AB, Amido Black; PAS, Periodic acid-Schiff; SBB, Sudan Black B.

MATERIAL AND METHODS

For cardiac-allatectomy only adult females of the American cockroach *Periplaneta americana* (L.) bearing oothecae were used. The cardiacum-allatum complex was carefully removed after ether anaesthesia through a slit made on the dorsum of the head behind the brain and between the compound eyes, taking care to see that the frontal ganglion and the oesophageal nerve were left intact. A few crystals of AMBISTRYN were placed in the wound, before it was closed with the original flap of the cuticle, and sealed with molten paraffin. Post-operative mortality was about 20% during the first week, but much less during the subsequent period. The animals were maintained on biscuits and water was provided *ad libitum.*

The neck was ligatured in a few animals bearing the ootheca, as this process is likely to induce a condition similar to that of cardiac-allatectomy. Their blood samples were analyzed after 4 days. Such animals showed some decrease in blood volume and, therefore, adequate amounts of hemolymph were obtained by centrifuging whole animals.

The hemolymph has been chosen for our investigations as it is a medium that is likely to reflect changes, if any, in protein metabolism. Blood samples were

TABLE I

Composition of hemolymph proteins in normal adult females of Periplaneta americana in relation to the ovarian cycle

Nature of the ovary or stage of reproduction	Protein fractions	Proteins (% of total distribution)	Lipids (% of total bound lipids)	Carbohydrates (% of total bound carbohydrates)
Average length of basal oocytes 2.70 mm.	1	5.38	6.77	—
	2	7.60	16.15	23.27
	3	52.94	72.92	46.82
	4	16.54	4.16	—
	5	6.18	—	29.91
	6	1.90	—	—
	9	9.46	—	—
Ootheca formed and tanned	1	5.55	13.19	12.50
	2	2.78	14.65	52.08
	3	35.42	72.16	—
	4	48.61	—	—
	5	3.12	—	35.42
	6	1.74	—	—
	9	2.78	—	—

taken from females 4, 7, 18, 30 and 60 days after the operation, and fractionated for proteins by disc electrophoresis in polyacrylamide gel columns as already described (Adiyodi and Nayar, 1966) using Amido Black (AB) as the staining dye. The success of the operation was tested by autopsy after bleeding, and the nature of the ovary noted in each case.

For the demonstration of glycoproteins the samples were electrophoresed as usual for 25 minutes and the gel columns then immersed in 7½% glacial acetic acid for 1 hour at room temperature. The columns were immersed in a 0.2% solution of periodic acid and placed in a refrigerator for an equal duration. Periodic acid was later removed electrophoretically for 1 hour using 7½% glacial acetic acid. The gel columns were placed in Schiff reagent and incubated in a refrigerator until the red bands became distinct. They were then removed and kept at room temperature in stoppered tubes.

Lipoproteins of the hemolymph were identified by staining the gel columns with Sudan Black B (SBB) in the manner described by Whittaker and West (1962).

Blood samples and tissue homogenates of fat bodies of normal adult females in different stages of vitellogenesis, homogenates of ovaries (average length of basal oocytes: 3.456 mm.) and blood samples of adult males were analyzed for information on conjugated proteins similarly by staining in periodic acid-Schiff reagent (PAS) and SBB. As far as possible electrophoresed blood or homogenate samples of the same individual were variously stained with AB, PAS, and SBB in one lot and this made easy the comparison of the hemolymph protein patterns, particularly of the conjugated proteins. The protein fractions in the stained gel columns have been numbered from the "origin," represented by the top of the small pore gel, to the "front," and their quantitation was done with a Canalco Model E Microdensitometer.

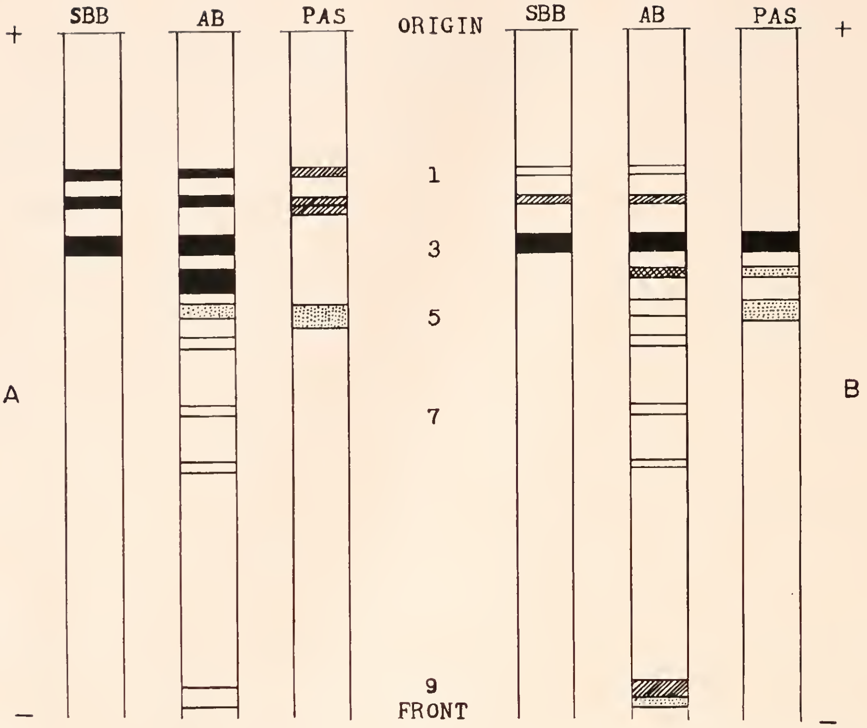

FIGURE 2. *Periplaneta americana.* Schematic representation of hemolymph proteins of (A) adult female with ootheca fully protruded and tanned, and (B) adult male. AB, Amido Black; PAS, Periodic acid-Schiff; SBB, Sudan Black B.

RESULTS

Nature of conjugated proteins in normal animals

In normal adult female cockroaches there are in all 9 detectable protein fractions stainable with AB in the hemolymph, 3 and 4 being the major proteins (Adiyodi and Nayar, 1966). In animals with protruding ootheca and also in their

TABLE II

Composition of hemolymph proteins in adult males of Periplaneta americana

Protein fractions	% of total distribution	% of bound lipids	% of bound carbohydrates
1	2.61	9.32	—
2	8.00	24.16	—
3	49.22	66.52	69.64
4	11.76	—	7.14
5	2.60	—	23.22
6	5.12	—	—
7	2.08	—	—
9	18.61	—	—

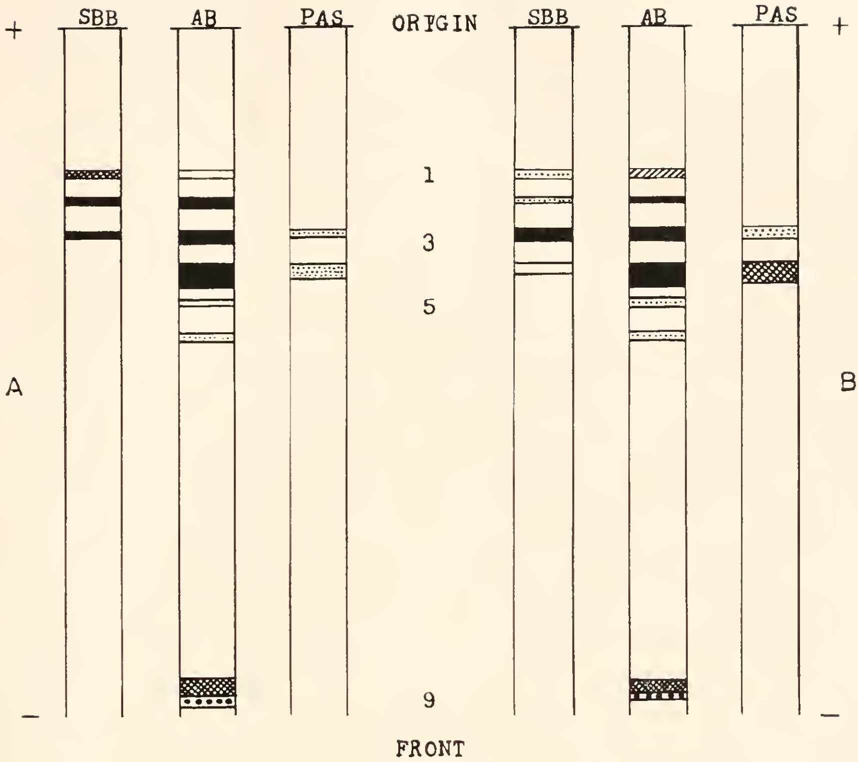

FIGURE 3. Pattern of hemolymph proteins of adult female *Periplaneta americana* 4 days after (A) Cardiac-allatectomy and (B) neck-ligaturing. AB, Amido Black; PAS, Periodic acid-Schiff; SBB, Sudan Black B.

pre-oviposition period, fractions 1 and 2 stain as lipo- and 2, 5 and 1 as glycoproteins (Figs. 1, 2). Fraction 2 generally and 1 often may, therefore, be said to occur as glycolipoprotein complexes.

Fraction 3 is a strongly indicated lipoprotein and contains, in the case of cockroaches constructing and bearing the oothecae, almost three-fourths of the bound lipids (Table I); during pre-ovipositional ovarian growth this appears to assume the characteristics of a glycolipoprotein complex, as it has been found to stain positively with PAS and SBB. The other fractions, including the female blood protein represented by fraction 4, reacted negatively with both the selective biochemical staining agents during periods of accumulation as while carrying the ootheca, but in the pre-ovipositional stages fraction 4 was found to show a mild reaction for lipids. Fraction 9, when present, was often bipartite and appears to represent some chromoprotein. The fat bodies of normal adult females taken at different stages of vitellogenesis gave uniformly a negative reaction with PAS and SBB; so did the homogenates of the ovary with yolk-laden eggs.

In males fraction 3 stains strongly positively with PAS and SBB, but contrary to the situation in females, fraction 4, which is present only in small amounts in

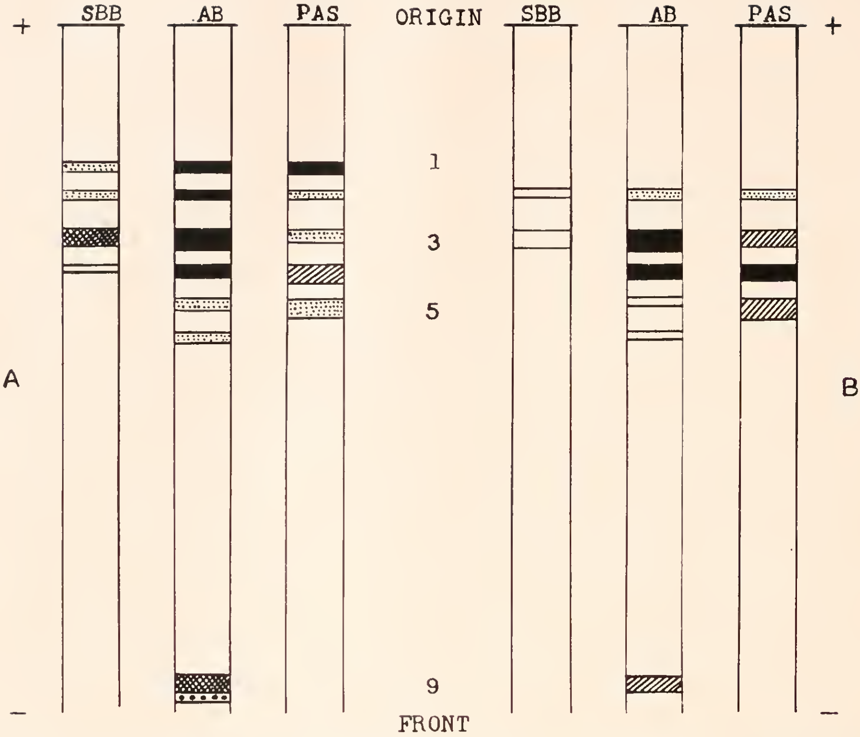

FIGURE 4. Electrophoretogram of hemolymph proteins of adult female *Periplaneta americana* (A) 7 days and (B) 18 days after cardiac-allatectomy. AB, Amido Black; PAS, Periodic acid-Schiff; SBB, Sudan Black B.

the male cockroach plasma, is non-sudanophilic, and reacts often with PAS (Fig. 2). Fraction 5 stains preferentially with PAS and has been found to contain more than one-fifth the net protein-bound carbohydrates (Table II) in the male hemolymph.

Pattern of hemolymph proteins in cardiac-allatectomized females

Cardiac-allatectomy in regular laying adult females suppresses ovarian activity by inhibiting vitellogenesis normally in about 7–12 days after the operation. Such females may produce either no ootheca at all, or deposit one or very rarely two of them before terminating the reproductive cycle. Rarely individuals have been found to deposit oothecae even 3–5 weeks after the operation; the number of oothecae deposited during this postoperative period, being few, in the order of 2 or 3. Sham-operated females did not differ from the normal either in the frequency of ootheca production or in their hemolymph protein patterns.

Figures 3–6 give a schematic representation of the hemolymph proteins of adult females 4, 7, 18, 30 and 60 days after cardiac-allatectomy. The experimental females exhibited uniformly an accumulation in fraction 4, and this tendency was quite evident even in animals examined four days after cardiac-allatectomy (Fig.

TABLE III

Effect of cardiac-allatectomy and neck-ligaturing on hemolymph proteins in adult female Periplaneta americana

Protein fractions	% concentration of the proteins					
	Neck-ligatured	Cardiac-allatectomized				
	4 days	4 days	7 days	18 days	30 days	60 days
1	1.79	1.83	3.67	—	4.82	9.86
2	6.74	7.83	4.42	7.54	7.27	9.42
3	7.49	9.46	52.84	46.54	45.94	47.98
4	65.87	56.75	27.74	33.62	33.57	26.01
5	4.49	0.78	2.27	1.80	3.36	3.59
6	2.69	2.61	2.27	1.50	3.91	2.25
9	10.93	20.74	6.79	9.00	1.13	0.89

3). Fraction 3, on the contrary, showed a marked decline in the amount of stainable material during the initial stage, much as in starved females (Adiyodi and Nayar, 1966). Thus in 4-day post-cardiac-allatectomized animals fraction 3 was nearly as extensive and strong as the minor band representing fraction 2. In 4-day experimentals fractions 1–3 stained for lipids and 3–4 for carbohydrates. Fraction 3 in these animals thus appears to be in the nature of a glycolipoprotein; it stained at this stage preferentially and strongly with SBB and gave only a comparatively mild reaction with PAS.

Seven-day post-cardiac-allatectomized females showed a conspicuous accumulation of stainable material in all fractions from 1–4 (Fig. 4). A bipartite "front" fraction (9) was conspicuous in gel columns stained with AB, much in the same form as in 4-day experimentals. Fraction 3 was stronger and more extensive and came to comprise as much as half the soluble proteins in the plasma, and this was attended with a corresponding decrease in the amount of stainable material in fraction 4. Fraction 3 had come to contain nearly two-thirds of the protein-bound lipid present in the hemolymph (Table IV). Fraction 4 also reacted positively though mildly with SBB. More striking, however, was the fact that all protein fractions from 1 to 5 gave a positive reaction for carbohydrates. If staining

TABLE IV

Distribution of protein-bound lipids in the hemolymph of cardiac-allatectomized adult female Periplaneta americana

Protein fractions	% concentration of protein bound lipids				
	4 days	7 days	18 days	30 days*	60 days
1	13.71	16.14	—	—	17.95
2	22.75	15.69	7.32	—	15.38
3	63.54	63.23	92.68	100.00	66.67
4	—	4.94	—	—	—

* Variable; for explanation see text.

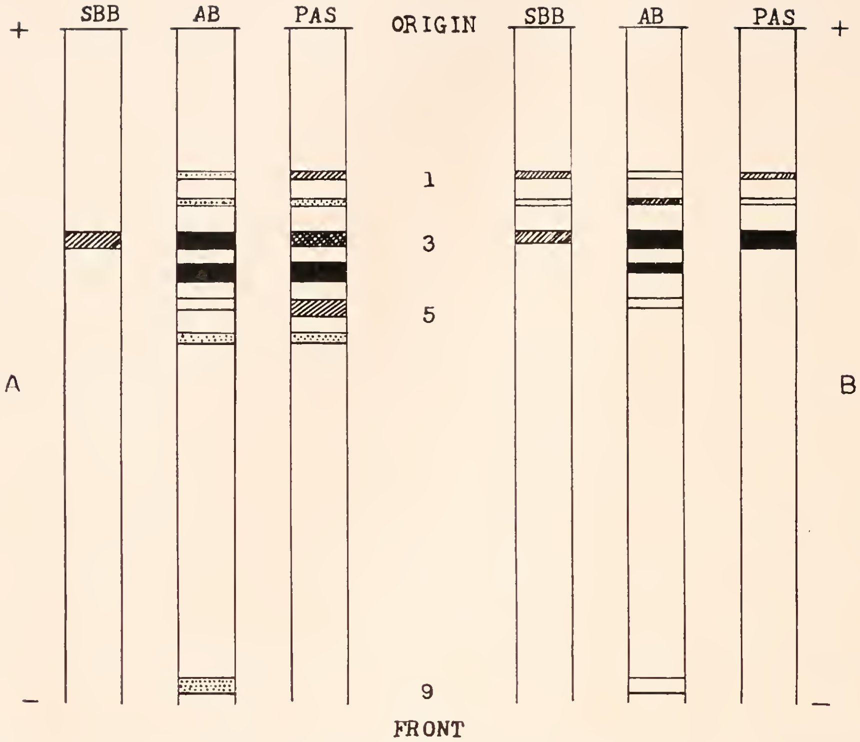

Figure 5. Disc electrophoresis of hemolymph proteins in 30-day post-cardiac-allatectomized adult females of *Periplaneta americana*. (A) with atrophied ovaries and (B) with basal oocytes 3.12 mm. in length. AB, Amido Black; PAS, Periodic acid-Schiff; SBB, Sudan Black B.

intensity with PAS and SBB is any criterion, respectively, of the concentration of the carbohydrate and lipid prosthetic groups within conjugated proteins, it may be said that the glycolipoprotein fraction 3 of 7-day post-cardiac-allatectomized females contained more lipid and less carbohydrate and conversely its fraction 4 more carbohydrate and much less lipid. There has thus been not only an increase in the number of bands reacting positively with PAS, but also a rise in the amount of material stainable with it.

The hemolymph pattern of 18-day post-cardiac-allatectomized females (Fig. 4) resembled that of 7-day animals in that there was nearly as much accumulation of AB-stainable material in fractions 3 and 4 (Table III) and also in that fraction 3 was likewise in the form of a glycolipoprotein complex. The slow moving fraction 1 was feeble or indistinct in AB, SBB and PAS preparations. Fractions 2–5 reacted with PAS, the most intense accumulation of stainable material being in fraction 4. Fractions 2 and 3 were sudanophilic, but only very mildly so, and contained, respectively, roughly only one-thirteenth and one-sixth the lipids normally bound in these fractions in ootheca-bearing phases.

In 30-day experimental animals all fractions from 1 to 6 have been seen to give

TABLE V

Distribution of protein-bound carbohydrates in the hemolymph of cardiac-allatectomized adult females of Periplaneta americana

Protein fractions	% concentration of protein bound carbohydrates				
	4 days	7 days	18 days	30 days	60 days
1	—	18.42	—	8.07	15.46
2	—	2.11	5.31	5.19	19.32
3	44.29	2.11	21.24	9.51	28.99
4	55.71	37.89	48.67	59.37	36.23
5	—	39.47	24.78	8.93	—
6	—	—	—	8.93	—

a positive and preferential reaction for glycoproteins, and fraction 3 and less frequently 1 and 2 reacted with SBB (Fig. 5). Judged by the intensity of staining with SBB, there appears to have occurred a slight accumulation of the lipid prosthetic groups in fraction 3 compared to 18-day animals. There was a considerable increase in the total amount of material stainable with PAS in the hemolymph, and fraction 4 came to contain as much as 59.37% of the total bound carbohydrates (Table V). This female fraction stained often as a strong glycoprotein with PAS even in those animals in which cardiac-allatectomy had been incomplete, with the anterior portions of the cardiaca remaining inadvertently unremoved during the operation. In a few of the females that showed varying degrees of oocyte growth 30 days after the removal of the cardiacum-allatum complex, fraction 4 was sometimes found to occur in the form of an unconjugated or lipo-protein and only fractions 1–3 stained for carbohydrates.

Experimental animals 60 days after the removal of the cardiacum-allatum complex (Fig. 6) still showed some retention of material in fraction 4, but only in nearly the same amounts as in 7-day post-cardiac-allatectomized animals. The fast moving "front" fraction (9) was almost inconspicuous. Fractions 1–3 reacted with SBB for lipids, 3 rather strongly so, and 1–4 with PAS for carbohydrates. Fraction 3 appeared bipartite with a proximal comparatively extensive staining area containing carbohydrates only in medium amounts and a narrow but densely PAS-positive disc-like part distally confluent with the former. In the electric field the mobility of this bipartite PAS-positive fraction has, however, been found to correspond well with that of fraction 3 in AB-stained gel columns.

Hemolymph proteins in neck-ligatured animals

Adult females with their necks ligatured and bled after 4 days showed slightly more protein in fraction 4 than 4-day post-cardiac-allatectomized females (Table III). This fraction in the former stained positively with SBB unlike that of the cardiac-allatectomized female. Neck-ligatured animals experienced difficulty in depositing ootheca and some of them even showed beginnings of ovarian malfunction. Accumulation in fraction 4 in the hemolymph of these animals is particularly interesting, because in the controls the stainable material in this fraction is low. In gel columns stained with AB, fraction 1 occurred as a weak band, but

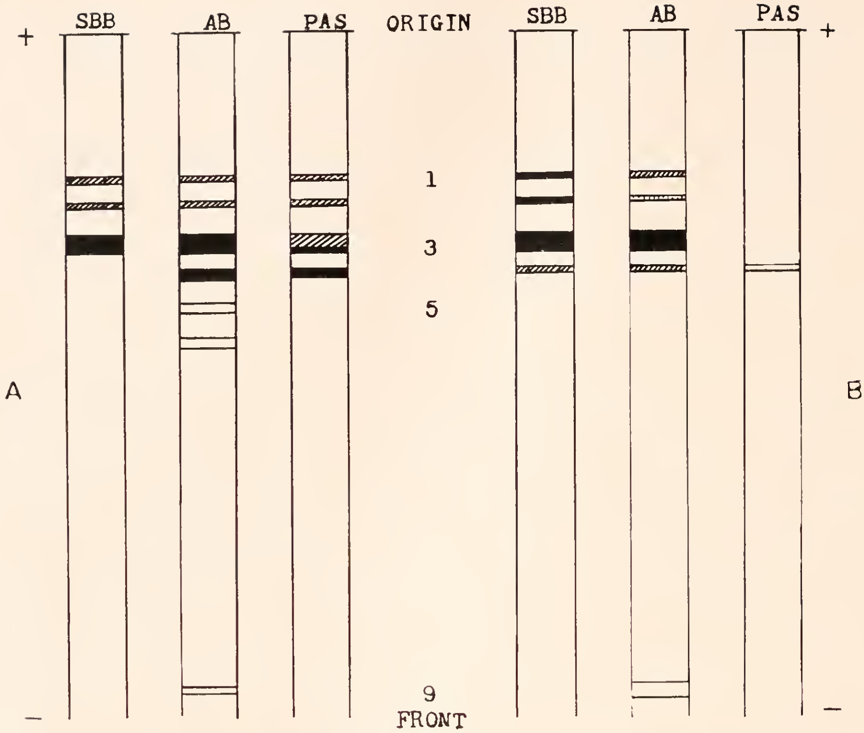

FIGURE 6. *Periplaneta americana.* Electrophoretogram of hemolymph proteins of (A) 60 lays post-cardiac-allatectomized adult female and (B) 21 days post-cardiac-allatectomized female, 3 days after implantation of cardiacum-allatum complex. AB, Amido Black; PAS, Periodic acid-Schiff; SBB, Sudan Black B.

the concentration in fraction 3 was almost comparable to that of 4-day post-cardiac-allatectomized animals. In both the cases there was also a strong "front" band representing fraction 9. Further, fraction 3 in both the cases was a glycolipoprotein complex; but fraction 4 reacted positively with PAS and SBB in neck-ligatured animals, whereas in cardiac-allatectomized females it was generally non-sudanophilic (Fig. 3). Fractions 3 and 4, the major proteins, together constituted only 66.21% of the total hemolymph proteins in 4-day post-cardiac-allatectomized females, but 73.36% in 4-day neck-ligatured animals. This increase may perhaps be related to inanition, the consequent general reduction in blood volume and the concentration of metabolites.

Effect of implantation of cardiacum-allatum complex in cardiac-allatectomized females

Though the reproductive ability of the female is impaired on removal of the cardiacum-allatum complex, such experimental females responded readily to the implantation of these organs and matured eggs in about 3–4 days, the normal time taken for a gonadal cycle by regular laying females. Thus 18-day post-cardiac-allatectomized females implanted with 3 cardiacum-allatum complexes, each taken from adult females, had basal oocytes measuring on the average 3.636 mm. in length when sacrificed 60–66 hours after the implantations.

Twenty-one days after the operation another set of animals were implanted similarly with 3 cardiacum-allatum complexes each and the blood fractionated for proteins 3 days afterwards. All the fractions from 1 to 4 were sudanophilic and fraction 4 alone showed any indications of carbohydrates. Fraction 4 occurred only in small concentrations, judged from its staining intensity in AB, PAS and SBB preparations (Fig. 6).

Discussion

The observations reported here point to the fact that correlated with different phases of ovarian activity there may be not only a change in the relative concentration of the different protein fractions, as already reported by us (Adiyodi and Nayar, 1966), but also differences in the chemical composition of the major proteins. Thus during ovarian growth fraction 3 is in the form of a glycolipoprotein complex, but when the ootheca has been fully formed and tanned, the fraction appears to be freed from the bound carbohydrates and gives a positive reaction for only the lipids. Similarly in the pre-oviposition period fraction 4 stains as a mild lipoprotein, but in females carrying the ootheca, and having this fraction accumulated in the hemolymph it appears to contain only protein and no stainable bound carbohydrates or lipids, as borne out by the negative reaction with SBB and PAS. The minor fractions, *viz.*, 1, 2 and 5, are a little variable in their occurrence, but when present are relatively stable as far as the staining affinities with the selective biochemical agents are concerned through the different stages of the ovarian cycle except perhaps fraction 1. Fraction 2, which is almost uniformly in the nature of a glycolipoprotein, and also 1 show a tendency for accumulation in the ootheca-bearing phase and stain more readily and intensely with SBB rather than PAS at nearly all the stages investigated. Fraction 5 is a monotonous and rather diffuse glycoproteinaceous band in both males and females, with its maximum concentration in ootheca-bearing females.

In 4-day post-cardiac-allatectomized females fractions 3 and 4 together constitute only 66.21% of the total hemolymph proteins, but in 7- and 18-day experimentals it is as much as 80% (Table III). This shows that in cardiac-allatectomized animals there is almost an immediate increase in the amount of proteins in these two electrophoretic components. Initially (*cf.* 4-day experimentals) there occurs a decrease in fraction 3 and a pronounced accumulation in fraction 4, but by a week after the operation the table is turned. But in 18-day animals the content of fraction 3 is again on a slight decline and 4 on the increase, compared to 7-day animals; with still further aging there occurs a reduction in the amount of AB-stainable material in fraction 4 (Table III).

Cardiac-allatectomy results not only in a pronounced accumulation of proteins in the blood, but also in changes in their chemical composition. In normal reproductive cycles, accumulation of the different protein fractions occurs during the ootheca-bearing phase, when fraction 3 stains as a lipoprotein and 4 reacts negatively with both SBB and PAS. In cardiac-allatectomized animals accumulation in 3 and 4 is nearly as pronounced as in ootheca-bearing animals (Tables I, III), and there is a tendency for both the fractions in the beginning stages to be in the nature of glycolipoprotein complexes (Fig. 4). With aging these two major proteins and also the other fractions stain preferentially for carbohydrates. The relatively slow-moving and usually dense-staining fraction 3 in our electrophoreograms seems to represent the "common insect protein" described by Whittaker and West (1962). A similar alteration in the composition of this common protein and the sex-specific protein has been reported in 14-day post-ovariectomized females of the same species by Thomas and Nation (1966a). They ascribed this phenomenon to the inability of the animal to maintain normal activity of the corpus allatum in the absence of the ovaries. In ovariectomized adult female cockroaches the corpus cardiacum and corpus allatum, in the absence of the normal feed-back from the ovary, become filled with large quantities of secretory material of extrinsic and intrinsic origin, respectively, and release of such materials appears to be considerably restricted (unpublished observations), a condition analogous in some way to cardiac-allatectomy, and whatever proteins synthesized under the influence of these factors may remain in the blood unutilized. Under conditions of allatectomy (Adiyodi and Nayar, 1966), ovariectomy (Menon, 1963, 1965; Thomas and Nation, 1966a) and cardiac-allatectomy there occurs an accumulation in the female blood protein (our fraction 4). Further, this fraction is present in homogenates of enlarged ovary (Adiyodi and Nayar, 1966) and shows variations in the hemolymph which could be clearly correlated with the reproductive cycle. Implantation of cardiacum-allatum complex in cardiac-allatectomized females results in a depletion of this protein already accumulated in the hemolymph and lifts the restraint on ovarian function. All these suggest strongly the possibility that the female blood protein is under the control of the cardiacum-allatum complex, and that it is normally removed from the blood by the ovary. The very similar biochemical behavior of the two major proteins in ovariectomized and cardiac-allatectomized females also suggests it as possible that in *Periplaneta americana* ovariectomy perhaps induces a state in the internal *milieu* physiologically comparable to that of cardiac-allatectomy.

The fast-moving fraction 9, generally found to occur in the blood as a strong bicolored band in cardiac-allatectomized adult females in their early stages and also in 4-day neck-ligatured females, reacts negatively with PAS and SBB, and seems to represent some chromoprotein. What probably looks like the pigment part of this protein(s) is inconsistent in its appearance in normal adult females, and we have not been able to correlate the presence or concentration of this fraction with any event in the reproductive cycle. However, under conditions of stress such as starvation (Adiyodi and Nayar, 1966) and cardiac-allatectomy it has been found that there occurs initially some accumulation of material in this conjugated fraction.

Mills, Greenslade and Couch (1966) observed some sort of a cyclicity in the RNA content of the fat body in the adult female American cockroach and suggest that the fat body may be contributing to hemolymph protein level in the early as well as the last phase of each gonadal cycle. But our fractionation studies on fat body homogenates of females in different stages of vitellogenesis seem to suggest, on the contrary, that none of the soluble lipo- or glycoproteins, including fraction 4, is probably as such synthesized or even stored in the fat body. No fractions other than 9, which appears to be only a tissue protein, could be detected by electrophoresing homogenates of this tissue of normal adult females and staining them with either of these three techniques.

Clark and Ball (1956) observed in *Periplaneta americana* lipid associated with each of the six protein fractions tested by them by paper electrophoresis, Stephen and Steinhauer (1957) only in two, and Siakotos (1960a) in 4 out of 5 fractions in the nymph. We have been able to detect lipid only in a maximum of 4 among the 9 fractions at any time during the reproductive cycle. In the case of the lipoproteins the lipid moiety appears to be contributed by the fat bodies to the proteins synthesized elsewhere, probably in the hemolymph itself. This view is in keeping with the suggestion of Gilbert, Chino and Domroese (1965) that triglycerides are synthesized and stored in the fat bodies and liberated into the blood as diglycerides, in which state they become bound to proteins for transport. Menon (1963, 1965) found a conspicuous fall in serum fats in allatectomized females, and it is now well known that the turnover of the triglycerides and phospholipids becomes affected in such animals (Vroman, Kaplanis and Robbins, 1965). Vroman, Kaplanis and Robbins (1965) further observed that 70% of the ovarian lipid is triglyceride and maintained on this ground that the allata regulate the metabolism of phospholipid and triglyceride in *Periplaneta americana* by exerting a control over the utilization mechanisms of these lipids. They ascribed the greater accumulation of the triglyceride fraction in allatectomized females to ovarian failure. We have at present no data regarding the nature of the lipid prosthetic groups of the different lipoprotein complexes in the cockroach plasma. However, the fact that the female blood protein (fraction 4) assumes the biochemical characteristics of a lipoprotein during ovarian growth in normal and also cardiacum-allatum complex re-implanted experimental females suggests the possibility that this fraction and also perhaps fraction 3 may serve normally as carrier proteins in the transport of the diglycerides in addition to the proteinaceous vitellogenic precursors to the ovary. Further, such a view will be also in agreement with the suggestion of Siakotos (1960a, 1960b) that the hemolymph proteins in the American cockroach act as carriers of nutrients such as carbohydrates and lipids in the molting process. It is also interesting to observe that the female sex-specific protein described by Siakotos (1960a) in the cockroach nymphs (his fraction III) is in the nature of a lipoprotein. In *Hyalophora cecropia* and *Antheraea polyphemus* the female protein is likewise sudanophilic (Telfer, 1965).

Fractions 3 and 4 found in the enlarged ovary of the American cockroach may represent vitellogenic blood proteins, as they appear in the ovaries only when laden with yolk (Adiyodi and Nayar, 1966). In females in advanced stages of vitellogenesis fraction 3 in the blood is in the nature of a glycolipoprotein complex and fraction 4 a lipoprotein, but in the homogenates of the ovaries of such animals these fractions are represented only as PAS- and SBB-negative simple proteins. In case the electrophoretic components 3 and 4 present in the enlarged ovary of the American cockroach owe their origin to similar fractions in the hemolymph rather than to independent synthesis [the ultrastructure of the ovarian surface is suggestive of one that could adsorb blood proteins (Anderson, 1964)], it may be said that the bound lipids and sugars become freed from these major proteins at entry.

Cardiacectomy interferes with protein metabolism (Bodenstein, 1953) and allatectomy with the synthesis and mobilization of proteins (Menon, 1963, 1965;

Adiyodi and Nayar, 1966; Thomas and Nation, 1966a) as well as lipids (Bodenstein, 1953; Menon, 1963, 1965; Vroman, Kaplanis and Robbins, 1965). It appears to be more than probable that the neurosecretory factors may reach the hemolymph through the cut ends of the cardiac nerves or other means in cardiacectomized or cardiac-allatectomized females, and that at least during the initial periods some protein may be synthesized under the influence of these factors as well as the allatal hormone already present. As most of the reserve fat occurs in the fat body in insects, analyses of whole-body fat as by Vroman, Kaplanis and Robbins (1965) may be considered as sufficiently indicative of the composition of fat in the fat body itself (see Kilby, 1963; Gilby, 1965). The accumulation of triglycerides (Vroman, Kaplanis and Robbins, 1965), therefore, in the fat body and the proteins in the hemolymph in allatectomized females is indicative of a mobilization failure. Serum lipid follows more or less the same cycle as that of the proteins (Menon, 1963, 1965), and the little available evidence seems to favor the view that lipid metabolism may be almost exclusively governed by allatal factors.

Information is scanty regarding the mechanism whereby the lipids combine with the lipoprotein protein: in mammals it has been suggested as probable that the lack in the synthesis of one moiety in this conjugated protein automatically affects the other (Robinson and Seakins, 1963). Robinson and Seakins found that treatment with chemical agents like ethionine, carbon tetrachloride and puromycin resulted in fatty livers in the rat and reduction in plasma lipid concentration, and they are of opinion that these changes in turn are caused by the inability of the liver to synthesize plasma lipoprotein protein. The relative loss of the lipid prosthetic group from hemolymph proteins in cardiac-allatectomized female cockroaches appears to be related to the bilateral arrest imposed on lipid and protein metabolism rather than on any one alone. An upset in the metabolism of carbohydrates may only be readily expected in our experimental animals as the operation involves removal of the corpora cardiaca. The reason for the preferential conjugation of several of the hemolymph protein fractions, particularly 4 to carbohydrate moieties and the consequent increase in the quantity of protein-bound PAS-stainable material in the hemolymph of cardiac-allatectomized animals, however, remains obscure. It is possible that in cardiac-allatectomized and allatectomized females the hydrolysis by active lipase of the triglycerides reported to be synthesized and stored in the fat body into diglycerides for release into the hemolymph becomes affected in the absence of the source of the allatal hormone. This may eventually upset the dynamic equilibrium between the fat bodies and the hemolymph. Such a view would also account for the increased storage of triglycerides (Vroman, Kaplanis and Robbins, 1965) in the fat body and the paucity of lipid fractions in the plasma proteins in the experimental animals. The marked loss in protein-bound lipids in the hemolymph of cardiac-allatectomized females may perhaps be related to their preferential utilization as an energy source, and the inability of the fat bodies to replenish the same due to arrest at some stage in their intermediary metabolism. Yet another possibility is that the vitellogenic blood proteins already synthesized, but not utilized, by the ovary become transformed into, or could probably be stored only as glycoproteins in the cockroach. Female American cockroaches administered crab eyestalk extract and having the ovarian growth retarded due to some ovary-inhibiting principle contained therein, showed a similar conversion of the unutilized female protein into a glycoprotein (Adiyodi and Adiyodi, unpublished). In this connection it may also be noted that the relatively small fraction 4, which constitutes about 11.76% of the total hemolymph protein of adult male cockroaches (Table II; Fig. 2), stains often as a glycoprotein. The larger conjugation to lipid prosthetic groups of fractions 1–3 which occurs in some females 30 and also 60 days after cardiac-allatectomy, compared to 18-day animals, may perhaps be related to some functional readjustment in lipid metabolism with further aging, but even in such animals fraction 4 usually remains a glycoprotein, when not utilized for oocyte growth. Starvation in the American cockroach is known to affect fraction 3, the "common insect protein" (Adiyodi and Nayar, 1966). Whittaker and West (1962) found similarly in last-instar *Malacosoma americanum* that this fraction almost disappeared on 48 hours of starvation. The conspicuous decrease in the concentration of fraction 3 in starved, neck-ligatured and cardiac-allatectomized females in the initial stages perhaps suggests that this "common insect protein" is in the nature of a nitrogen reserve hydrolyzed to maintain amino acids, and thus the osmotic equilibrium under stress conditions (see Wyatt, 1961; Loughton and West, 1965). Restitution of this fraction takes place probably by conversion of fraction 4 into proteins of lower electrophoretic mobility, as borne out by a decline in the concentration of fraction 4 with aging in starved as well as cardiac-allatectomized females.

SUMMARY

1. The nature and behavior of the conjugated proteins in the hemolymph of adult female *Periplaneta americana* (L.) have been studied in relation to the ovarian cycle, using disc electrophoresis in polyacrylamide gel and various staining procedures. It is shown that there may be not only a change in the relative concentration of the different protein fractions with oocyte growth, but also differences in their chemical composition. Fraction 3 and the female fraction 4, which represent the major proteins in the blood, appear to serve as carriers of lipids to the ovaries, besides most likely providing proteinaceous yolk precursors to the same. Fractionations made with ovarian homogenates seem to indicate that the lipids and sugars bound to such proteins may become freed at entry into the ovary.

2. Cardiac-allatectomy has been found to result in a pronounced accumulation of proteins in the blood, and also in changes in their chemical composition. In such animals there is a tendency with aging for the loss of lipid prosthetic groups and for several of the fractions including the major proteins to stain preferentially for carbohydrates. This effect could be reversed by the implantation of fresh cardiacum-allatum complexes taken from adult females. It is suggested that in cardiac-allatectomized females there is probably a bilateral arrest of lipid and protein metabolism, and that the vitellogenic proteins already synthesized, but not utilized by the ovary, become converted into glycoproteins.

3. Fractionation studies on fat body homogenates of females in different stages of ovarian activity appear to indicate that in *Periplaneta americana* none of the soluble lipo- or glycoproteins, including the female fraction, is as such synthesized or stored in the fat body.

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NUCLEIC ACID AND PROTEIN SYNTHESIS IN THE DEVELOPING OOCYTES OF THE BUDDING FORM OF THE SYLLID, AUTOLYTUS EDWARSI (CLASS POLYCHAETA)[1]

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In recent years the importance of the role of deoxyribonucleic acid (DNA) and the various ribonucleic acids in protein synthesis and the significance of the origin of new proteins in cellular differentiation have become increasingly clear. It now seems an accepted fact that the nucleus is the major site of ribonucleic acid (RNA) synthesis in a wide variety of cell types, including developing oocytes (Ficq, 1961; Ficq, *et al.*, 1963; Favard-Séréno and Durand, 1963a). However, as far as intranuclear metabolism is concerned, the situation is far from clear. Favard-Séréno and Durand (1963a) observed that the chromosomes are the primary site of RNA synthesis in follicle cells of the cricket ovary; from its source RNA then migrates to the nucleolus where it accumulates before moving to the cytoplasm. Presumably the nucleolus in these cells is serving simply as a temporary storage depot for RNA. However, for a wide variety of cells, and this includes a number of types of developing oocytes (for example, Ficq, 1953, 1955a, 1955b; Edström, 1960; Edström *et al.*, 1961), the conclusion has been that the nucleolus is an important site of RNA synthesis providing a major portion of cytoplasmic RNA.

One approach to the problems of whether the nucleolus is simply accumulating RNA and whether or not RNA synthesis is correlated with DNA and protein synthesis in developing eggs has been the use of radioautography. Various investigators (for example, Brachet and Ficq, 1956; Ficq, 1959) have employed specific radioactive precursors to determine the site of synthesis of RNA, DNA, and proteins within developing oocytes, and to follow the subsequent migration of these macromolecules. The oocytes thus far investigated have been mainly those of amphibians (Ficq, 1955a; Ficq *et al.*, 1958; Pantelouris, 1958; Gall, 1963; Ozban *et al.*, 1964), echinoderms (Ficq, 1955b, 1962; Geuskens, 1963, 1965), and insects (King and Burnett, 1959; Nigon and Nonnenmacher, 1961; Favard-Séréno and Durand, 1963a, 1963b; Vanderberg, 1963), and, more recently, of an echiuroid worm (Das *et al.*, 1965), and two species of polychaetous annelids (Tweedell, 1964, 1966; Allen, 1966).

The developing oocytes of the polychaete, *Autolytus edwarsi,* which were used in the present investigation, incorporate uridine-H^3 with unusual rapidity (Allen, 1966), and also incorporate phenylalanine-H^3. It was thus felt that the details of the results on *Autolytus* eggs should be reported, and particularly in view of the fact that varying results have been reported as to the site of RNA and protein synthesis in unfertilized eggs.

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[1] This research was supported by grants G-19958 and GB-2853 from the National Science Foundation.

The following description will serve as background for the radioautographic study. The writer recently has pointed out that "The Syllidae are unusual among the polychaetes in that some genera of this family reproduce both asexually by the formation of stolons from a parent stock, and sexually by the union of gametes from male and female individuals which, in turn, originate from stolons produced asexually" (Allen, 1964, page 187). The genus *Autolytus* is a good example of this type of life cycle. The eggs of the budding form of *A. edwarsi* develop in a rather unusual manner (Gidholm, 1963). Gidholm confirmed Meyer's discovery (1914) that ovaries occur only in the stock of *Autolytus* yet the whole chain of stolons, even the undifferentiated ones, may contain well developed eggs. Gidholm points out that Meyer "correctly interpreted these facts to mean that the eggs are not formed in the stolons but rather in the posterior part of the stock, from where they are transported to the stolons" (Gidholm, 1963, page 529). According to Gidholm, the oocytes are released from the ovary, not into the coelom as Meyer had thought, but into an epithelial-lined oocyte cavity which is continuous from the ovaries of the stock through the region of proliferation to the budding stolons. By vital staining of the oocytes, Gidholm was able to follow migrating eggs from the oocyte cavity of the stock through the region of proliferation where the cavity narrows to a canal, and then into the differentiating stolons in which the continuous oocyte cavity enlarges again (Gidholm, 1963, page 533, Figs. 1 and 2, and page 538).

The budding form of *A. edwarsi* with its linear chain of successively differentiating stolons provides an ideal place for the study of cellular differentiation; it is small enough so that in the present study the whole organism (stock with attached stolons) was treated with labeled precursors for nucleic acids and proteins. Since the oocytes are the largest cells and thus lend themselves to more accurate cytological analysis than do the other cells, the results presented first are those demonstrating incorporation of labeled precursors into developing eggs.

The results indicate that RNA is synthesized (or accumulated) in the nucleolus of the developing oocytes from which it migrates to the rest of the nucleus and then to the cytoplasm (also see Allen, 1966). Protein synthesis, on the other hand, appears to occur throughout the developing oocyte (*i.e.*, in the nucleolus, the non-nucleolar regions of the nucleus, and the cytoplasm). There was no evidence of incorporation of thymidine into the developing oocytes, indicating the absence of any appreciable DNA synthesis.

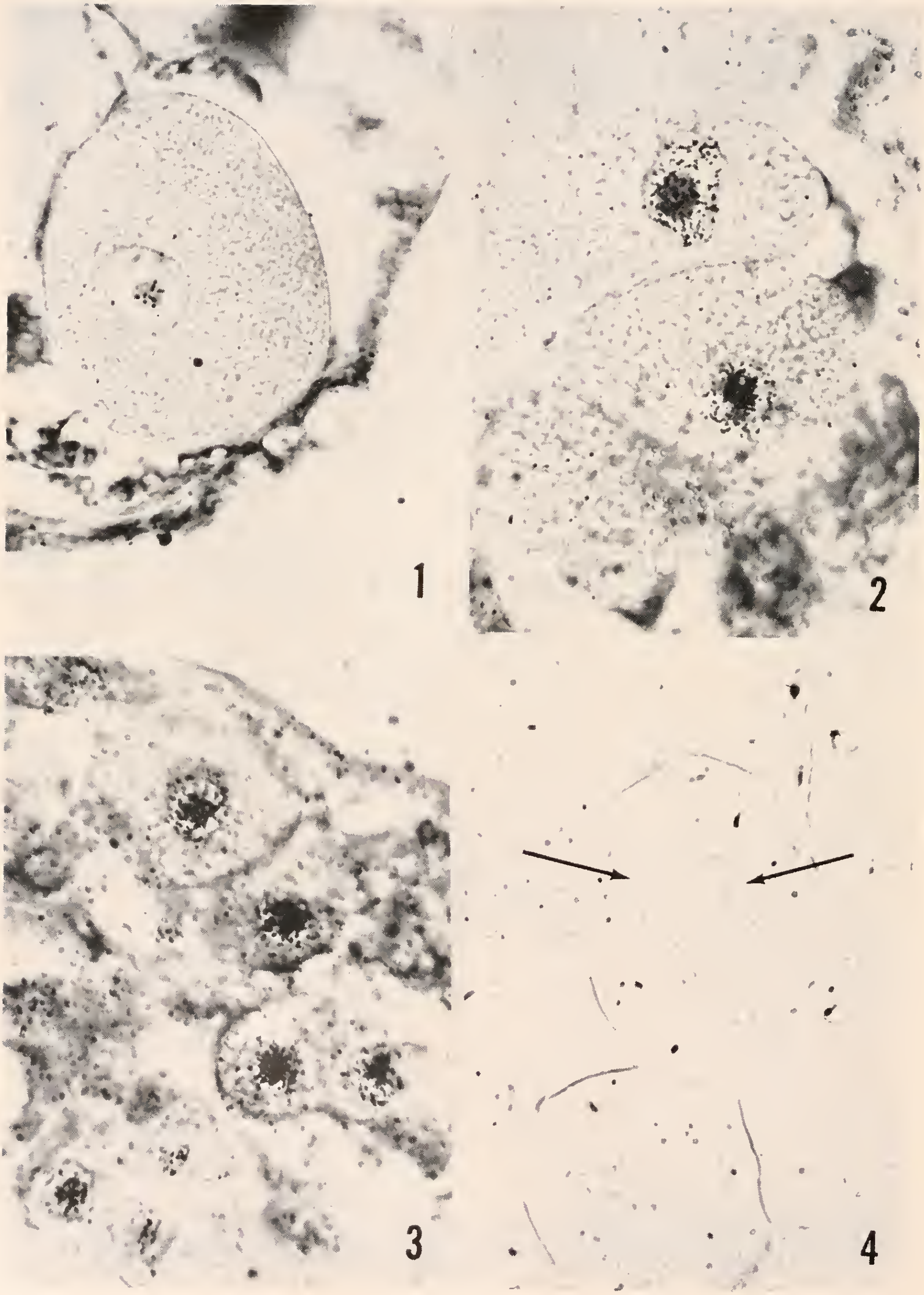
Material and Methods

The budding form of *Autolytus edwarsi* lives in association with the hydroid, *Laomedea (Obelia) geniculata*, which in turn, is found abundantly on the brown alga, *Laminaria*. Stocks of *A. edwarsi* with linear chains of stolons, and stocks which were not obviously budding, were kept until needed in small covered glass dishes supported on a perforated tray suspended in the sea water aquarium. Preliminary experiments in which stocks with their attached stolons were immersed in the isotope solutions indicated that these multicellular organisms were small enough so that very short periods of treatment were sufficient to permit diffusion of radioisotopes through their body wall. Tritiated uridine, thymidine, and phenylalanine were used to determine the site of synthesis (or accumulation) of RNA, DNA, and protein, respectively, in the tissues of the stock and differentiating stolons of *A. edwarsi.* The treatment with radioisotopes, subsequent fixation of the worms, and embedding in paraffin were done at the Kristineberg Zoological Station, Sweden, in the fall of 1963.

In general, 10 stocks with their differentiating stolons were treated at a time in one milliliter of a solution of radioisotope in filtered sea water. During exposure to the isotope, the cover of the glass dish in which they were treated was lined with a layer of filter paper moistened with distilled water to prevent evaporation and maintain the proper humidity. Worms were immersed thus for periods varying from 2 minutes to 20 hours in one of the following radioisotopes: 2.5 μc./ml. of uridine-H^3 (0.90 c./m*M*, Schwarz Bioresearch, Inc.), 2.5 μc./ml. of thymidine-H^3 (6.05 c./m*M*, Schwarz Bioresearch, Inc.) or approximately 2.5 μc./ml. of DL-phenylalanine-H^3 (33.2 mc./m*M*, New England Nuclear Corporation). In early experiments, following exposure to the isotope, the worms were washed in several changes of sea water (200 ml. for each change), but in later experiments they were placed in an excess of non-labeled precursor, *i.e.*, uridine, thymidine, or DL-phenylalanine, in filtered sea water. The treated animals were fixed in four changes of acetic-alcohol (1:3) of approximately 10 minutes each, at room temperature in the earlier experiments but at refrigerated temperature in later ones (the lower temperature decreased the amount of muscular contraction). The fixative was washed out in four changes of 70% ethanol; the last change was overnight at 4° C. The animals were then dehydrated and embedded in paraffin. Serial sections were cut at 5 μ. Control sections were extracted as follows: (1) worms treated with uridine-H^3: slides of tissue sections were extracted in ribonuclease (Worthington, 0.02% at pH 6.5 for one to two hours at 37° C.); (2) worms treated with thymidine-H^3: tissues sections were extracted in deoxyribonuclease (Worthington, 0.02% or 0.04% in 0.003 *M* $MgSO_4$ at pH 6.5 for one to two hours at 37° C.); (3) worms treated with DL-phenylalanine-H^3: (a) a few slides were placed in ribonuclease as in (1) above; another group was placed in deoxyribonuclease as in (2) above; (c) some tissue sections were digested in deoxyribonuclease as in (2) followed by ribonuclease as in (1); (d) other tissue sections were hydrolyzed in 5% TCA at 90° C. for 30 minutes to remove both DNA and RNA.

After the extractions, in making the first sets of radioautographs, both extracted sections and those not digested with enzymes were placed in dilute solutions of the appropriate non-labeled precursor (uridine, thymidine, or DL-phenylalanine) in distilled water and air-dried. For the rest of the radioautographs, extracted sections and those not digested with enzymes were treated for 5 minutes in 5% trichloracetic acid (TCA) at 2–4° C. to remove acid-soluble nucleotides. Sections were then passed through three changes (10 minutes each) of cold 70% ethanol and air-dried. Some of the sections of worms treated with thymidine-H^3 (including samples of sections digested in deoxyribonuclease and non-extracted sections) were hydrolyzed in 1 N HCl and stained with Feulgen; slides were subsequently air-dried from 70% ethanol.

The dried slides were then placed in distilled water in a water bath at 44–45° C. and coated with Kodak NTB3 liquid emulsion. After drying, slides were placed



FIGURES 1–4.

in sealed black boxes at 4° C. Following various exposure times, coated slides were developed in D-11 and air-dried.

The sections of budding worms which had been treated with thymidine-H^3 and stained with Feulgen were then either mounted directly with euparal or were first counterstained with 2% acidified fast green, and mounted in Canada balsam. The remaining sections of animals treated with thymidine-H^3, and most of the sections of animals treated with uridine-H^3, were stained with 0.025% azure B in pH 4 McIlvaine's buffer at 37° C. for periods up to 15 minutes. After staining with azure B (Woodard, Rasch and Swift, 1961) most of the slides were rinsed in distilled water for several minutes, air-dried (this avoided the white precipitate formed after tertiary butyl alcohol) and mounted in euparal. The rest of the sections of animals treated with uridine-H^3, and the majority of sections treated with DL-phenylalanine-H^3, were stained for 15 to 30 seconds in 0.1% toluidine blue at pH 6, rinsed in 95% ethanol, air-dried, and mounted in euparal.

The remainder of the sections of worms treated with DL-phenylalanine-H^3 were stained one hour in 1% aqueous fast green at pH 2 (Woodard, Rasch and Swift's modification, 1961, of Schrader and Leuchtenberger, 1950).

Results

In radioautographs, silver grains over a particular structure were interpreted as an indication of incorporation of the radioisotope precursor into that structure and as signifying synthesis; the presence of silver grains after short pulses may indicate initial synthesis, after longer pulses may indicate transfer or accumulation as well as synthesis.

RNA synthesis. In the developing oocytes of *Autolytus edwarsi*, incorporation of uridine-H^3, as evidenced by overlying silver grains, was noted primarily in cell structures which were shown cytochemically to contain RNA. Developing oocytes of stolons which had been treated with uridine-H^3 for two to fifteen minutes show incorporation of the radioisotope primarily over nucleoli (Fig. 1). In two stolons, some of the eggs after a 5-minute pulse and 10-minute pulse, respectively, had a few silver grains over the non-nucleolar portion of the nucleus although most of the grains were concentrated over the nucleolus. Other eggs of these two stolons

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All figures are radioautographs of sectioned material which had been coated with Kodak NTB3; the photomicrographs were all taken at the same magnification with Contrast Process Ortho film.[2]

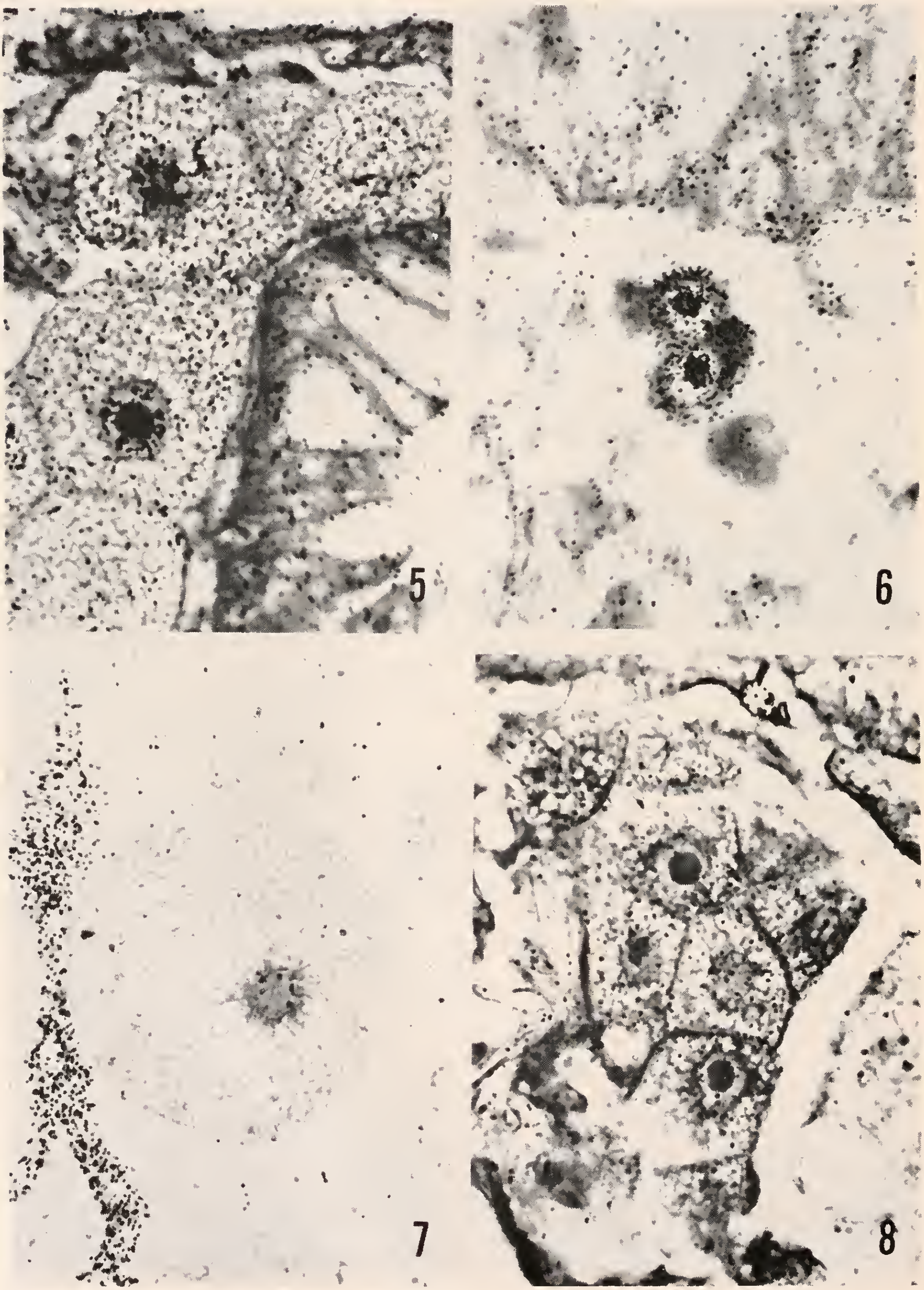
FIGURE 1. A large oocyte given a 10-minute pulse of uridine-H^3; exposure, 14 days; stain, azure B at pH 4. Silver grains over nucleolus only.

FIGURE 2. Two oocytes given a 30-minute pulse of uridine-H^3; exposure, 14 days; stain, azure B at pH 4. Silver grains over non-nucleolar regions of the nucleus as well as the nucleolus.

FIGURE 3. Oocytes given a 2-hour pulse of uridine-H^3; exposure, 3 days; stain, azure B at pH 4. Silver grains over the nucleoli and non-nucleolar regions of the nuclei, and beginning to appear over the cytoplasm.

FIGURE 4. RNase-digested control for oocytes shown in Figure 2; stain, azure B at pH 4. The arrows indicate the diameter of the nucleus. Note the absence of silver grains other than background, as well as the absence of color.

[2] The first three figures were published by the writer's permission in Dr. J. R. Collier's review article in "Current Topics in Developmental Biology," Vol. I, 1966, Academic Press, New York.



Figures 5–8.

showed no grains other than background over any part of the egg. Radioautographs of eggs exposed to uridine-H^3 for 30 minutes show grains over the nucleolus and over the rest of the nucleus (Fig. 2). In Figures 1 and 2 the eggs had the same exposure time so the number of grains indicates the relative amount of RNA synthesis. Oocytes exposed to the radioisotope for two hours show a few grains over the cytoplasm as well as over the nucleolus and non-nucleolar regions of the nucleus (Fig. 3). Further increase in length of exposure to uridine-H^3 resulted in an increased number of silver grains over the cytoplasm (Fig. 5), especially in the case of younger eggs (Fig. 6). This difference between older and younger eggs is even more marked than the photomicrographs suggest as the younger eggs received a shorter pulse (9 hours instead of 16 hours) and a shorter exposure period (3 days instead of 8 days) than did the older oocytes. In radioautographs of eggs pre-digested with RNase and stained with azure B, the color reaction for RNA was absent and very few silver grains were present (Fig. 4). These results were interpreted as showing that the most rapid synthesis of RNA occurs in the nucleolus of the developing oocytes of *A. edwarsi*, from which it migrates to the rest of the nucleus and then to the cytoplasm. The presence of the few grains over the non-nucleolar regions in some eggs after 5-minute and 10-minute pulses may indicate synthesis of chromosomal RNA or, as compared with other eggs, a precocious migration of nucleolar RNA into the nucleoplasm. Figures 3 and 5 indicate that only slight transfer of nuclear RNA has occurred within two hours and that only moderate transfer has been accomplished after 16 hours (some of the cytoplasmic grains in Figure 5 are background). After a 20-hour pulse, results are similar to those observed after a 16-hour pulse. Thus the nucleolus appears to be extremely active in RNA synthesis during the growth period of oogenesis, and migration of RNA to the cytoplasm is a relatively slow process, at least during the latter part of the growth period.

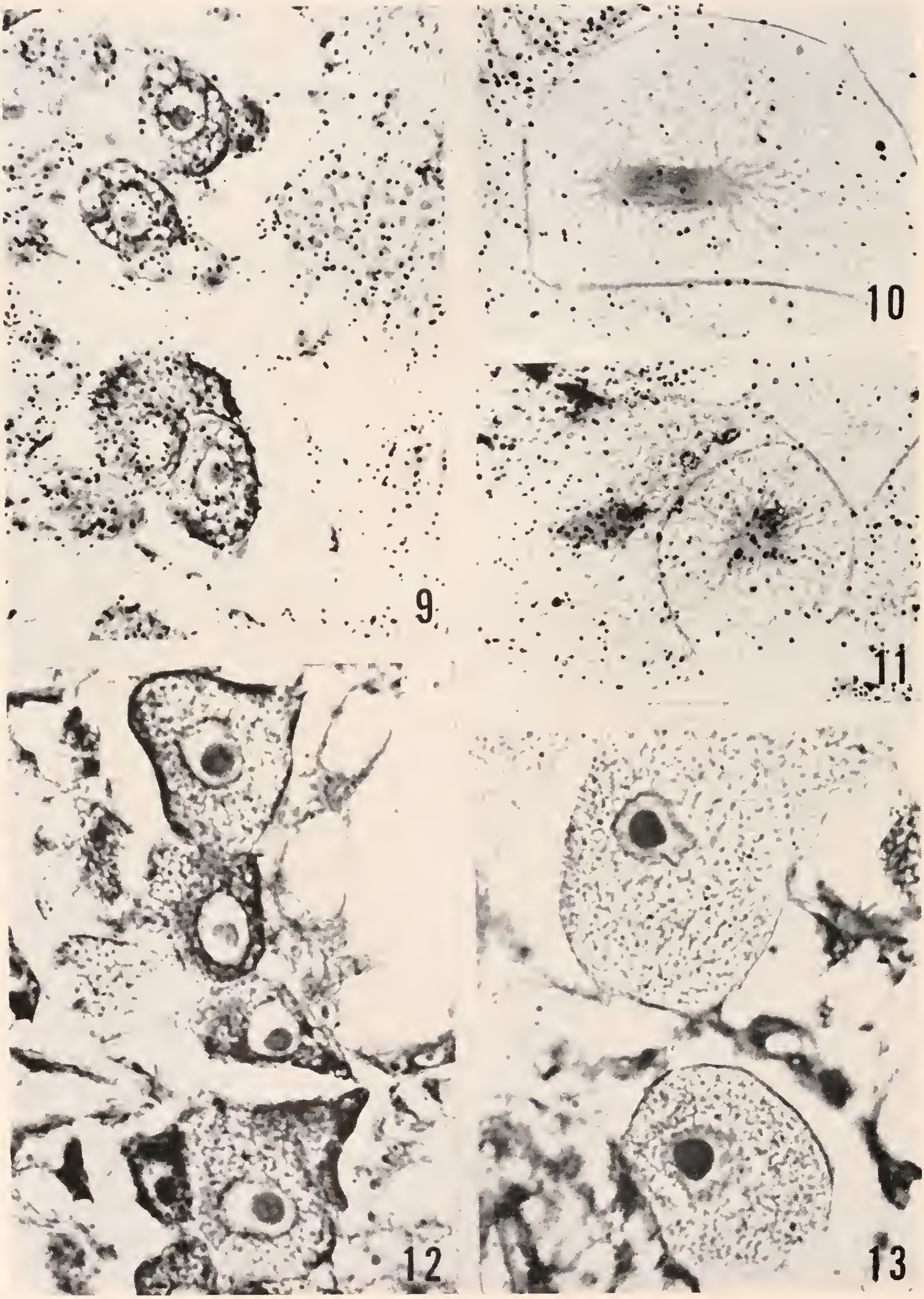
A sacconereis stage with the body cavity packed with fully grown oocytes in metaphase I (the eggs of *Autolytus* are fertilizable at this stage; Allen, 1964, and Gidholm, 1965) was given a 20-hour pulse of uridine-H^3. The results are illustrated by Figure 7. Of particular interest is the absence of grains (other than background) over all parts of the eggs. Thus, provided the permeability of the egg to uridine has not changed, it would appear that no significant RNA synthesis is occurring in fully grown eggs that are ready to be fertilized; this suggests that the synthetic machinery for RNA is turned off by the end of the growth period in this species.

FIGURE 5. Oocytes given a 16-hour pulse of uridine-H^3; exposure, 8 days; stain, azure B at pH 4. Silver grains over the nucleoli and the non-nucleolar regions of the nucleus, and more obvious over the cytoplasm than in Figure 3.

FIGURE 6. Two young oocytes given a 9-hour pulse of uridine-H^3; exposure 3 days; stain, azure B, pH 4. In comparison with Figure 5, the silver grains are more concentrated over the cytoplasm.

FIGURE 7. A fully grown oocyte at metaphase I (polar view showing 6 chromosomes) given a 20-hour pulse of uridine-H^3; exposure, 3 days; stain, azure B, pH 4. Note the absence of silver grains other than background.

FIGURE 8. Oocytes given a 30-minute pulse of phenylalanine-H^3; exposure, 21 days; stain, azure B, pH 4. Note that silver grains are scattered over nucleoli, non-nucleolar portions of nuclei, and cytoplasm.



Figures 9–12.

Protein synthesis. The number of budding worms treated with phenylalanine-H^3 was smaller than in the case of uridine-H^3, and very few of those treated had large oocytes. Following pulses of 30 minutes to 20 hours, radioautographs showed scattered silver grains over all parts of the developing eggs. Extraction with ribonuclease, deoxyribonuclease or hot TCA did not prevent fast green from staining the eggs, nor did it reduce the number of silver grains; therefore, it was assumed that phenylalanine-H^3 was being incorporated specifically into proteins. As shown in Figures 8 and 9, there are scattered grains over all parts of the developing eggs with no concentration of grains over the nucleolus. It thus seems apparent that all parts of developing oocytes (nucleoli, non-nucleolar regions of the nucleus, and cytoplasm) are synthesizing proteins, and that the nucleolus is no more active than any other part of the egg in this synthesis.

An older female stolon which had been given a 20-hour pulse was found to have the body cavity packed with a mixture of fully grown oocytes and cleavage stages (presumably fertilization had occurred accidentally, as normally in *Autolytus* fertilization and early development take place in a ventral egg sac or brood pouch; Gidholm, 1965, and Allen, 1964). Figures 10 and 11 are photomicrographs from the same radioautograph. Figure 10 shows an undivided egg in metaphase I; Figure 11 shows a cleavage stage in metaphase (some of the grains are background). A comparison of the two stages shows that the fully grown unfertilized egg is synthesizing little, if any, protein. In contrast, the grains over the cytoplasm of the cleaving blastomeres indicate that protein synthesis is occurring. Of particular interest, however, is the concentration of grains over the nuclear regions and spindles of cleaving blastomeres and the absence of grains other than background over the spindles of unfertilized eggs. Thus it seems clear that proteins for the mitotic spindles are being synthesized during cleavage whereas the proteins for the meiotic spindles are synthesized prior to the maturation divisions.

DNA synthesis. Budding female stocks at various stages of differentiation were treated with thymidine-H^3 following pulses of five minutes to twenty hours; radioautographs of the stolons showed no silver grains other than background over the eggs. Figures 12 and 13 show radioautographs of developing oocytes following a 60-minute pulse. The absence of silver grains indicates that the oocytes of *A. edwarsi*, whether they are large or small, do not incorporate thymidine-H^3. This is not because this precursor of DNA was not diffusing to them, for aggregates of silver grains (indicating incorporation and hence synthesis of DNA) were observed over nuclei in parapodia and other tissues in the same radioautographs.

FIGURE 9. Young oocytes given a 60-minute pulse of phenylalanine-H^3; exposure, 60 days; stain, toluidine blue at pH 6. Even with a longer pulse and longer exposure period than the eggs shown in Figure 8, silver grains are still not concentrated, but are scattered over the entire oocyte.

FIGURES 10 AND 11. A fully grown unfertilized oocyte (Fig. 10) and a cleavage stage (Fig. 11) from the same radioautograph; both were simultaneously given a 20-hour pulse of phenylalanine-H^3; exposure 6 days; stain, azure B, pH 4; some of the silver grains are background. In comparison with the younger oocytes in Figures 8 and 9, note the scarcity of grains over the fully grown oocyte. In comparison with the cleavage stage (Fig. 11), note the absence of silver grains other than background over the meiotic spindle, and the few grains in the cytoplasm.

FIGURES 12 AND 13. Oocytes in different stages of growth, all from the same stolon, which were given a 60-minute pulse of thymidine-H^3; exposure 7 days; stain, azure B, pH 4. Note the absence of silver grains over all parts of the eggs, whether the oocytes are large or small.

That no significant synthesis of DNA was occurring in these oocytes is borne out by the staining reactions. DNA was demonstrated by Feulgen and azure B in various tissues of the stocks and differentiating stolons but not in developing eggs. Apparently this nucleic acid is too dispersed in developing oocytes of this polychaete to give a positive reaction with the cytochemical methods used. Earlier, this was found to be the case in fully grown oocytes of *Diopatra* (another polychaete); the writer, however, successfully demonstrated the presence of DNA by staining with gallocyanin-chromalum (Allen, 1961). In the case of *Autolytus* eggs, tests for DNA were negative following gallocyanin-chromalum. Judging, then, from radioautographs and cytochemical tests, the DNA in the germinal vesicle of *Autolytus edwarsi* is diffusely distributed and presumably was synthesized very early in oogenesis.

Discussion

The preferential uptake of uridine into the nucleolus of *Autolytus* oocytes is in essential agreement with the results from other eggs (Tweedell, 1966; Piatigorsky *et al.*, 1967). RNA synthesis is more rapid during the earlier part of oogenesis, while there is no significant synthesis of RNA in the fully grown egg; however, the absence of information concerning uptake of uridine by the unfertilized egg prevents one from drawing a definite conclusion (compare, for example, Piatigorsky and Whiteley, 1965; Gould, 1965; Siekevitz *et al.*, 1966). Thus at the end, or near the end, of the growth period, genetic transcription may cease in *Autolytus*.

The most rapid synthesis of RNA in *Autolytus* oocytes occurs in the nucleolus from which it migrates to the rest of the nucleus and then to the cytoplasm. Recently, Penman *et al.* (1966) have demonstrated in HeLa cells that all of the 45S precursor to ribosomal RNA is contained in the nucleolus and that this precursor yields a 16S RNA that is rapidly transferred to the cytoplasm, as well as a 35S fragment that remains in the nucleus for a short time. This 35S portion subsequently fragments to produce 28S ribosomal RNA that moves into the cytoplasm. The RNA in *Autolytus* oocytes which accumulates in the nucleolus and moves from there to the rest of the nucleus and then to the cytoplasm fits the pattern of transfer of ribosomal RNA in HeLa cells. Thus the RNA synthesized in *Autolytus* eggs may represent different types of ribosomal RNA. There is evidence that other oocytes (particularly their nucleoli) synthesize, or accumulate, relatively large amounts of ribosomal RNA (Davidson *et al.*, 1964; Brown, 1964, 1966; Brown and Littna, 1964b, 1966; Gross *et al.*, 1965; Vincent *et al.*, 1966).

From other work, it is known that messenger RNA is also produced in developing oocytes (Slater and Spiegelman, 1966; Davidson *et al.*, 1966; Crippa *et al.*, 1967), and probably transfer RNA (Vincent *et al.*, 1966). Most radioautographic studies, including the present one on *Autolytus,* provide insufficient evidence for determining whether or not the RNA that is rapidly synthesized in developing oocytes represents one or more types of RNA; however, the fact that protein synthesis is also occurring in *Autolytus* oocytes indicates that messenger RNA is produced during oogenesis.

The developing oocytes of *Autolytus* synthesize their own RNA during the growth period of oogenesis instead of relying on nurse cells. This is in contrast to the developing eggs of the polychaete, *Diopatra,* in which cytochemical evidence suggests that RNA is passed from nurse cells across a cytoplasmic bridge to the developing oocyte (Allen, 1961). It is also in contrast to the developing eggs of certain insects which apparently are furnished at least part of their RNA by follicle cells, or by specialized nurse cells (Sirlin and Jacob, 1960; Favard-Séréno and Durand, 1963a; Vanderberg, 1963).

Favard-Séréno and Durand (1963a) observed that in the cricket ovary it is the chromosomes of the follicle cells that are the primary site of RNA synthesis. From its source RNA then migrates to the nucleolus where it accumulates before moving to the cytoplasm. It was pointed out in describing radioautographic results for *Autolytus* that the eggs of a few stolons are exceptions in having silver grains over the non-nucleolar regions of the nucleus after a pulse of 5 to 10 minutes. Differences in permeability of stolons of different ages might account for the variation or, as compared with the majority of eggs, the exceptions may indicate a precocious migration of nucleolar RNA into the nucleoplasm due to a variation in synthetic rates among nucleoli. Another possible interpretation is that RNA is being synthesized by the chromosomes and is migrating in the reverse direction, *i.e.*, from chromosomes to nucleolus, as in the follicle cells of the cricket ovary. Should this be the case, one must still assume that the nucleolus in these oocytes represents an extremely active site of RNA synthesis; otherwise, uridine would not be incorporated so rapidly into the nucleoli of the majority of oocytes. It is also possible that transfer in both directions is occurring; *i.e.*, that some grains over the non-nucleolar portions of the nucleus may indicate RNA migrating from chromosomes to the nucleolus, while others may represent RNA migrating from the nucleolus to the cytoplasm. The results of the present study suggest that the major movement would be the latter.

Protein synthesis. The evidence provided by the present investigation indicates that all parts of the developing oocytes of *A. edwarsi*—*i.e.*, nucleolus, the rest of the nucleus, and cytoplasm—synthesize proteins. There was no preferential localization of silver grains; in other words, the nucleolus did not appear to be a major source of synthesis (or accumulation) of proteins.

In contrast to the non-localized uptake of protein precursors in *Autolytus* oocytes, Ficq (1953, 1955a, 1955b) demonstrated that the nucleolus of starfish and amphibian oocytes is more active than the rest of the egg in incorporating glycine and phenylalanine, suggesting a higher degree of protein synthesis in the nucleolus. Later work on amphibian oocytes demonstrated that phenylalanine was incorporated uniformly in the cytoplasm, and also labeled the nucleus (Brachet and Ficq, 1956; Ficq *et al.*, 1958). Pantelouris (1958), using *Triturus* oocytes, demonstrated that the site of uptake of protein precursors and the direction of their migration within developing oocytes depends on whether or not vitellogenesis is occurring. Ficq, Pavan and Brachet (1958) had concluded that the nucleus, particularly the lampbrush chromosomes, may be active in synthesizing proteins. More recently, Gall (1963) demonstrated in oocytes of the newt that the lampbrush chromosomes incorporate phenylalanine throughout the length of their loops, thus providing evidence that these chromosomes are active in protein synthesis.

Brachet (1960) points out that radioautographic studies indicate that the nucleolus play a more conspicuous role in RNA synthesis than in protein synthesis. One can conclude from the radioautographs of *Autolytus* that RNA is being synthesized more rapidly than proteins in the nucleoli of developing eggs. It has been demonstrated for the clawed toad that a marked synthesis of ribosomal RNA occurs during oogenesis (Brown, 1964), and that new ribosomal RNA does not occur until the beginning of gastrulation (Brown and Littna, 1964a). This suggests that the ribosomal RNA stored during oogenesis is the only ribosomal RNA available for protein synthesis up to the time of gastrulation. Perhaps the transfer of rapidly synthesized RNA from nucleolus to cytoplasm in *Autolytus* oocytes means that messenger and/or ribosomal RNA is being stored during oogenesis for later use, *i.e.,* for early embryonic development.

The radioautographic observations on *Autolytus* eggs indicate that no significant incorporation of DL-phenylalanine-H^3 is occurring in fully grown oocytes in metaphase I, while during cleavage incorporation does occur, being heaviest over the nuclear and spindle regions. A preferential incorporation of DL-leucine-H^3 into mitotic spindles of early cleavage stages has been demonstrated in the sea urchin (Gross and Cousineau, 1963; Gross, 1964), the label being highly selective for the spindles and nuclei. The evidence from radioautographs of *Autolytus* suggests that in these eggs also the proteins for the mitotic spindles are being synthesized during cleavage. In contrast, it would appear that proteins for the meiotic spindles are synthesized prior to the maturation divisions so presumably they are simply assembled during meiosis. It is well established that fully grown unfertilized eggs of the sea urchin show little metabolic activity but that a marked protein synthesis begins almost immediately after fertilization (Hultin, 1950, 1961; Giudice, *et al.*, 1962; Tyler, 1963; Gross, 1964); some of these new proteins are thus required for mitosis (Hultin, 1961; Gross, 1964).

DNA synthesis. As noted above, the evidence presented indicates that the developing oocytes of *A. edwarsi* do not incorporate thymidine-H^3. It thus seems evident that no significant DNA synthesis is occurring during the growth period of oogenesis, either in the developing germinal vesicle or in the cytoplasm. It was concluded, therefore, that nuclear DNA synthesis must occur very early in oogenesis. For another polychaete, *Pectinaria,* Tweedell (1966) recently demonstrated that nuclear uptake of thymidine-H^3 is restricted to the early period of oogenesis, *i.e.*, before the growth of the oocyte begins. Similarly, in various other species studied, the evidence shows that thymidine-H^3 is not incorporated into the nucleus during the growth period of the egg. This is true for oocytes of the sea urchin (Ficq *et al.,* 1963), the cricket (Favard-Séréno and Durand, 1963b), and the mouse (Mintz, 1964). Nor is thymidine incorporated into the germinal vesicle of the fully grown oocyte in most species studied (Ficq, 1961; Simmel and Karnofsky, 1961; Nigon and Nonnenmacher, 1961; Favard-Séréno and Durand, 1963b; Ficq *et al.,* 1963). Probably in all these forms synthesis of nuclear DNA occurs early in oogenesis as has been demonstrated for the mouse egg (Rudkin and Griech, 1962). Recently, in the adult promsimian, *Galago demidoffi,* Ioannou (1967) demonstrated incorporation of thymidine-H^3 by oocytes at pre-leptotene or leptotene as well as by oogonia in prophase. These developing germ cells of the adult ovary thus are capable of synthesizing DNA but whether they ever become functional eggs has not been determined.

In *Autolytus* eggs no cytoplasmic synthesis of DNA was evident. Tweedell (1966), however, was able to demonstrate cytoplasmic uptake of thymidine-H^3 in developing *Pectinaria* oocytes, suggesting the synthesis of cytoplasmic DNA in these eggs. Pikó, Tyler and Vinograd (1967) recently have demonstrated DNA in mitochondria and yolk spheres of sea urchin eggs. They cite a number of papers that demonstrate that large amounts of cytoplasmic DNA occur in the mature eggs of many species of animals. For some oocytes, it has been shown that the associated follicle cells contribute to ooplasmic DNA. Incorporation of thymidine occurs in the nuclei of follicle cells in the cricket with a subsequent transfer of radioactive DNA into their cytoplasm and into the ooplasm (Favard-Séréno and Durand, 1963b). The possibility still exists that cytoplasmic DNA occurs in *Autolytus* oocytes but since no incorporation of thymidine was evident, it appears that no DNA synthesis was taking place during oogenesis.

The writer gratefully acknowledges her indebtedness to Dr. B. Swedmark for his many courtesies while she was carrying out this research at Kristineberg Zoological Station, and to Dr. D. M. Prescott for introducing her to the techniques of radioautography. She also wishes to express her appreciation to Dr. J. R. Collier for his critical reading of the manuscript and for his helpful suggestions.

SUMMARY

1. The incorporation of tritiated uridine, thymidine, and phenylalanine into nucleic acids and proteins was studied in the developing oocytes of stolons produced by asexual budding in *Autolytus edwarsi*.

2. Extremely rapid incorporation of uridine-H^3 into the nucleolus of developing oocytes suggests the importance of the nucleolus in the synthesis of RNA. Oocytes exposed to uridine for successively longer periods than two to fifteen minutes show silver grains over the non-nucleolar regions of the nucleus as well as the nucleolus, and finally a number of grains appear over the cytoplasm (the relative number of cytoplasmic grains is greater in younger oocytes). Results indicate that RNA is synthesized mainly in the nucleolus of developing oocytes from which it migrates to non-nucleolar regions of the nucleus and then to the cytoplasm. Transfer of RNA to the cytoplasm is a relatively slow process in these eggs.

3. Fully grown oocytes at metaphase I (the stage at which *Autolytus* eggs are fertilizable) do not incorporate uridine-H^3, indicating that no significant synthesis of RNA is occurring at the close of the growth period of oogenesis.

4. Phenylalanine-H^3 was incorporated into all parts of the developing oocytes, and was not incorporated more rapidly into the nucleolus. Thus it seems apparent that all parts of developing eggs (nucleoli, non-nucleolar regions of the nucleus, and cytoplasm) are synthesizing protein. Fully grown oocytes in metaphase I, however, are synthesizing little, if any, protein. In contrast to ripe eggs, cleavage stages are synthesizing proteins, particularly in nuclear and spindle regions. The results for cleavage stages are similar to those of the sea urchin. The results for *Autolytus* suggest that in these eggs the proteins for the mitotic spindles are being synthesized during cleavage whereas the proteins for the meiotic spindles are synthesized prior to the maturation divisions.

5. Thymidine-H^3 was not incorporated into developing oocytes, indicating that no significant DNA synthesis was occurring. Radioautographs and cytochemical tests indicate that DNA is diffusely distributed in the germinal vesicle of *Autolytus edwarsi*. Presumably in this species, DNA is synthesized very early in oogenesis.

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AUTORADIOGRAPHIC AND CYTOLOGICAL STUDY OF BLASTODERMAL CELLS IN TURKEY EGGS SUBJECTED TO EXTENDED PRE-INCUBATION STORAGE[1, 2]

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The length of the interval between oviposition and start of incubation is the most important single factor that affects the subsequent developmental history of the turkey gastrula: prolonged storage progressively reduces the early embryo's capacity to re-initiate and maintain ontogenesis (Kosin and Mun, 1965). Cytological evidence, based on the observations of chicken blastoderms (Arora and Kosin, 1967), suggests that cells of the early avian gastrula continue to exhibit some mitotic activity at temperatures even as low as 7.2° C. However, the same study has shown that the mitotic cycle of these cells is incomplete—it does not proceed beyond metaphase. The objective of the present study, reported below, was to extend similar cytological observations, augmented by the use of tritiated thymidine, to cells of turkey gastrulae, both during pre-incubation storage and the first hours of post-storage incubation.

MATERIALS AND METHODS

Two genetically distinct lines of Broad Breasted Bronze turkeys were used in the study, both originating in 1954 from a single heterogeneous population. Of the two, Line 1 was a random-bred population while the other, Line 3, was single-trait-selected for high egg production. Although Line 3 turkeys, at the time the data were collected, were characterized by a substantially higher rate of egg production than Line 1, with respect to body size and hatchability, both lines were equal. The eggs were gathered daily, at approximately hourly intervals, between 9:00 AM and 4:30 PM from single-sire pens. All eggs were identified according to the hens that laid them. Shortly after 5:00 PM the eggs were transferred from the pens to a holding room maintained at 13° C. and 80% relative humidity. During storage, the eggs were kept with the large (blunt) end up.

Series 1. After overnight cooling, Line 1 eggs were allotted randomly to two groups: control and experimental. In the control fertile eggs, the germ discs were removed and fixed for cytological examination. The fertile eggs in the experimental group, after 3 days of storage, were injected with tritiated thymidine, sealed, and stored for an additional 6 days. The injection procedure was as follows: the shell was cleaned with 70% ethanol, and a small window (1 cm.^{2}) was cut in the large end of the egg. (Because the egg had been kept throughout in the vertical position, the blastoderm was usually found to be located just under the air-cell.)

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The cut piece of the shell and its membrane were then reflected and the solution containing the isotope was injected, directly under the blastoderm, with a 1.0-ml. micrometer syringe, equipped with a 12-mm. 27-gauge needle, the tip of which was slightly turned up to facilitate the delivery of the solution under the blastoderm. The total volume injected per blastoderm was 0.05 ml. containing 1.6 μc. of T-H^3, with specific activity of 6.64 μc./μ*M*. The solution containing the isotope was held at 13.0° C. Following the injection, the cut piece of the shell and its membrane were reflected back into place, the opening was sealed with paraffin and plastic tape, and the eggs returned to storage at 13.0° C.

After the storage period, the treated eggs were removed and blastoderms fixed for 4 hours in a 19:1 mixture of absolute ethanol and acetic acid at −6° to −8° C., according to the method described by Wolman and Behar (1951). This was followed by dehydration in absolute ethanol at room temperature overnight. The blastoderms then were cleared in xylene, embedded in paraffin and cut serially at 7 μ. For comparative purposes, adjacent sections from different regions of the experimental and control blastoderms were mounted on the same slide. After staining, which involved the Feulgen-Fast Green reaction, the slides intended for the preparation of autoradiographs were held in water at 40–45° C. before the diluted emulsion was applied to the slides. The emulsion (NTB—Kodak) was diluted 1:2 with distilled water at 40–45° C., and the slides were dipped twice, individually, and then allowed to stand on end in a rack until the emulsion dried completely (*cf.* Messier and Leblond, 1957). The slides were placed in plastic boxes containing Drierite, sealed with black tape and stored in the refrigerator (4–5° C.) for 20–28 days. Care was taken that the slides were in a horizontal position in the box, with the emulsion side down. Following this exposure, the slides were developed for 3 minutes in Kodak D-19 at 18° C., rinsed in tap water, and fixed for 6 minutes in Kodak Acid Fixer and then washed in running tap water for 10–15 minutes. Finally, the slides were dehydrated in ethanol, cleared in xylene and mounted in Permount.

The determination of the frequency of mitotic, necrotic, and labeled nuclei was based on counts within at least 30 random unit areas from various sections of each blastoderm. The unit area measured approximately 0.48 × 0.07 mm. The values for these three parameters were expressed per 100 nuclei examined. Morphologically, some necrotic nuclei appeared pycnotic, while others became much enlarged. They were all characterized by loss of structural detail and deep staining following the Feulgen reaction. All nuclei showing a grain count above the level that could be ascribed to background fogging were considered to be labeled. To be counted as "mitotic," the nuclei could be in any stage of the mitotic cycle.

Series 2. Line 3 eggs were allotted randomly to storage treatments of 1, 7, 14, or 21 days' duration at 13.0° C. and 80% relative humidity. Following storage each egg received the injection of a saline solution containing T-H^3. The injection procedure followed the general plan described for the experimental eggs in Series 1, except for volume of the solution and concentration of T-H^3: 0.07 ml. of the solution containing 7.77 μc. of the isotope at the specific activity of 9.2 μc./μ *M*. After the injection, the eggs were incubated for 10 hours. At the end of this period the blastoderms were removed, fixed and sectioned serially at 5 μ. Adjacent sections from different regions of each blastoderm were mounted on the same slide.

TABLE I

Frequency (%) of mitotic and necrotic cells in the blastoderms of ovipositol and stored eggs. Series 1

Category	Mitotic cells	Necrotic cells
Ovipositol (non-stored)		
$\bar{x}$	5.2	1.9
Range	(1.1–9.3)	(0.4–3.9)
Stored		
$\bar{x}$	5.6	2.9
Range	(1.7–9.5)	(1.1–5.4)

Thus two slides, I and II, were prepared from each blastoderm. All slides were subjected to the Feulgen-Fast Green staining reaction. Prior to the preparation of autoradiographs, the I and II slides were treated as follows:

I: These were stained and mounted permanently for histo-cytological studies. They were not exposed to the emulsion.

II: After the usual steps involving xylene, a descending series of ethanol and, finally water, the slides were treated for 5 minutes with 5% TCA at 4° C. The slides were then stained and allowed to dry at room temperature. A few hours before the application of the emulsion, the slides were dipped in a 1% collodion solution for a few seconds (prepared in a 1:1 mixture of ether and absolute ethanol) and allowed to dry in the air (*cf.* Gross *et al.*, 1951, and Kopriwa and Leblond, 1962). From then on the procedures for the preparation of autoradiographs and subsequent cytological analysis were the same as outlined earlier in Series 1.

RESULTS

The data concerning the frequency of mitotic and necrotic nuclei in the ovipositol (*i.e.* non-stored) and Line 1 blastoderms are summarized in Table I. The fre-

TABLE II

Frequency (%) of labeled nuclei in blastodermal cells of stored eggs, exposed to tritiated thymidine. Series 1

Blastoderm no.	Frequency of labeled nuclei
1	2.5
2	1.1
3	2.2
4	1.6
5	1.3
6	0.0
7	0.9
8	1.8
9	1.0
10	2.1
11	1.7
12	0.0
$\bar{x}$	1.6

TABLE III

Frequency (%) of $T\text{-}H^3$ *labeled nuclei in the blastoderms of eggs subjected to different pre-incubation storage periods, followed by 10 hours of incubation. Series 2*

Storage length (days)			
1	7	14	21
3.14[a]	3.21	0.91	0.24
2.64	4.10	0.56	0.00
1.02	0.92	2.98	2.10
3.19	3.12	1.21	0.00
4.21	0.98	3.46	1.20
3.21	2.98	2.98	1.12
1.92	0.94	0.29	0.42
2.62	4.10	4.21	2.81
4.12	0.92	0.82	0.00
5.21	....	1.10	0.00
4.00	....	....	....
$\bar{x}$ 3.21	2.30	1.85	0.69[b]
			1.15[c]
Range 1.02–5.21	0.92–4.21	0.29–4.21	0.00–2.81

[a] Each value represents the frequency of $T\text{-}H^3$-labeled nuclei in a single blastoderm.

[b] Based on total number of blastoderms involved.

[c] Based on the number of embryos showing labeled nuclei.

quency value in each category was based on 12 blastoderms. The data show that there was an increase in the frequency of these nuclei in the blastoderms stored for extended period, indicating that mitotic activity continued during storage at 13° C. Although the differences were not large, their consistency was unmistakable. The majority of the mitotic figures was associated with the epiblast. By contrast, the necrotic cells were largely found in the hypoblast and in the zone of junction.

The autoradiographic studies demonstrated the incorporation of tritiated thymidine into some of the nuclei of the stored blastoderms. The frequency of the nuclei labeled with $T\text{-}H^3$ in the stored blastoderms is summarized in Table II.

The incorporated $T\text{-}H^3$ was observed in interphase and early prophase, sometimes in metaphase, but never in anaphase. In general, the frequency of $T\text{-}H^3$-labeled nuclei was low.

Table III shows the frequency of nuclei labeled with $T\text{-}H^3$ in the blastoderms incubated for 10 hours following different pre-incubation storage treatments of Line 3 eggs (Series 2). It will be seen that the frequency of nuclei decreased markedly when the length of storage was more than 7 days. Among the labeled cells, most were in interphase or in early prophase, although some were in metaphase. Not all the mitotic figures observed in sections were labeled. Moreover, there was extreme individual variation among blastoderms with respect to the incidence of labeled nuclei, particularly among the blastoderms of eggs stored for 14 and 21 days. In the latter group, 4 blastoderms of the 10 examined were devoid of labeled nuclei. In these blastoderms, and in a few from other treatments, the evidence of radioactivity was either exclusively or largely localized around the yolk granules. Such blastoderms lacked the sub-germinal cavity.

Table IV

Frequency (%) of mitotic, fragmented, and necrotic nuclei in the blastoderms of eggs subjected to different pre-incubation storage periods, followed by 10 hours of incubation. Series 2

Storage length (days)	Type of nuclei		
	Mitotic	Fragmented	Necrotic
1	5.26	1.81	3.58
7	4.54	2.68	3.94
14	2.83	5.74	4.90
21	2.63	5.15	8.22

Data bearing on the further cytological examination of the slides which were not exposed to the emulsion (Slide I) are presented in Table IV. The frequency value in each classification was based on 10–12 blastoderms. One sees from it that the frequency of fragmented nuclei increased as the storage was extened to 14 days or beyond, whereas the opposite was true for mitotic figures, indicating an inverse relationship between these two parameters. The fragmented nuclei were characterized by the presence of both large and small, usually round, masses of nuclear material which stained deeply with nuclear dyes. These "micro-nuclei" were found either enclosed within the intact nuclear membrane or within the cellular plasm in the cells in which the nuclear membrane could no longer be detected.

In addition, the blastoderms from eggs first stored for extended periods and then incubated for 10 hours revealed the presence of various types of nuclear irregularities. Among these the most frequent were: tripolar mitosis, amintosis and chromosomal aberrations such as double chromatid bridges.

Discussion

An earlier report from this laboratory (Arora and Kosin, 1967) has shown that the "physiological zero" for chicken blastoderms (when the term is used to designate the absence of activity recognizable at the intracellular level with the aid of a light microscope) is below 13° C., the temperature recommended for holding hatching eggs before incubation. Some mitotic activity continued even when eggs were kept at 7.2° C., for as long as 21 days. In the present study, based on turkey blastoderms, the experimental design called for a single pre-incubation temperature at 13° C. Keeping this point in mind, the parallelism of results obtained in the comparable phases of the two studies is striking. In both, the mitotic activity in aging blastoderms was blocked at metaphase: no cells were observed to proceed with mitosis beyond that stage during the experimental holding period. Consequently, progressive lengthening of storage was accompanied by an accumulation of "blocked" cells.

The existence of mitotic activity in blastodermal cells at 13° C. was corroborated by the observation, in the present study, that the DNA synthesis continued within such cells. Evidence for the incorporation of tritiated thymidine in the nuclei was unequivocal, although the frequency of cells involved in the process was low. The presence of mitotic figures devoid of the isotope among the cells of blastoderms which received T-H^3 early in the storage period suggests an explanation for two possible conditions which are not necessarily mutually exclusive: one is that the cells in mitosis were present *before* the initiation of "quiescent" (storage) period and that they remained in that stage; the other is the availability of the isotope to some of the cells at least was reduced by physical means. For example, the frequently observed absence of the sub-germinal cavity in the early gastrulae of eggs exposed to 37.5° C., following an extended period of temperature-induced quiescence, indicates that the liquefaction of the yolk under such blastoderms does not proceed at a normal rate. It is possible that the solution containing the isotope, when injected under the blastoderm of this type, was prevented from reaching the physiologically active cells of the epiblast. This would, of course, introduce an error in the subsequent cytological analysis of the physiological state of the affected blastoderm.

The aging process in the avian blastoderm is characterized by a steep rise in the frequency of necrotic nuclei. No direct evidence is available on the possible relationship between cells blocked at metaphase and those classified as "dead." However, the close and consistent association between their respective frequencies strongly indicates that necrotic cells may be a by-product of blockage. As suggested earlier (Arora and Kosin, 1966a, 1966b, 1967), this chain of events on the cellular level may be responsible for the reduced viability, during subsequent incubation, of chicken and turkey blastoderms previously subjected to extended storage. Support for this comes from the present study: following pre-incubation storage, the turkey blastoderm upon being exposed to 37.5° C., optimal for normal embryogenesis, responds by a decreased level of T-H^3 labeling of the nuclei and an increased frequency of fragmented nuclei and of mitotic irregularities.

SUMMARY

An autoradiographic and cytological study involved blastoderms of Broad Breasted Bronze turkey eggs subjected to storage up to 21 days at 13° C. and 80% relative humidity. In one phase of the study, the blastoderms were treated with tritiated thymidine during storage; in the other phase, the blastoderms were first stored, then treated with T-H^3 and, finally, incubated for 10 hours at 37.5° C.

Results indicate that:

1. The frequency of mitotic and necrotic cells in the blastoderms increased during pre-incubation storage.

2. Nuclei, labeled with T-H^3, were found present in the blastoderms exposed to the isotope, both during and after storage.

3. The "aged" blastoderms, when incubated for 10 hours, showed a high incidence of necrotic nuclei, of nuclear fragmentation, of mitotic irregularity, and a decreased frequency of labeling with T-H^3.

The conclusions reached are that:

1. The turkey blastoderm is physiologically active during extended storage at 13° C.: it exhibits evidence of some DNA synthesis and of undergoing limited mitosis.

2. The accumulation of cells blocked at metaphase during storage may be a major factor responsible for the subsequent moribundity of such blastoderms.

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EXCYSTATION OF APOSTOME CILIATES IN RELATION TO MOLTING OF THEIR CRUSTACEAN HOSTS.
II. EFFECT OF GLYCOGEN[1]

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The apostomes are an order of ciliates symbiotic with Crustacea, being dormant and encysted on their host's exoskeleton for most of their life cycle. All apostomes of the family Foettingeriidae are reported to excyst when their hosts molt (Chatton and Lwoff, 1935). The exuvial fluids trapped in the host's cast-off exoskeleton are the only food of many species (the exuviotrophs). Other genera (the histotrophs) also excyst at the death by injury of their hosts and feed upon the tissue fluids of the corpse.

Excystation is always preceded by an extensive metamorphosis of the encysted stage, the phoront, involving its general body shape, organization, infraciliary pattern and extensive changes in its physiology (Chatton and Lwoff, 1935; Bradbury and Trager, 1967). The metamorphosis prepares the phoront (a non-feeding stage) for the rapid ingestion and concentration of a large volume of food. At the same time the phoront's ciliature is modified and augmented so that later the engorged ciliate (the trophont), swollen to 30 times its initial volume, is still able to swim. The histotrophic phoronts undergo their metamorphosis within a few hours of settling on their hosts. The exuviotrophic phoronts can remain dormant for weeks or months and only metamorphose immediately before the molting of their hosts.

The exoskeletons of Crustacea do not completely seal the animal from its external environment. The exoskeleton is traversed by pore canals and joined by sutures which weaken during the pre-molt period. Food substances—glycogen, lipids, and proteins—build up in the blood and tissues during the premolt stage of the molt cycle (Passano, 1960; Martin, 1965). The possibility therefore exists that some substance (or substances), that increases in concentration in the blood and tissues of pre-molting crabs, leaks out in increasing amounts as the molt approaches and stimulates metamorphosis and excystation of the apostome ciliate. The following experiments were designed to test this possibility.

MATERIALS AND METHODS

Several hundred hermit crabs (*Pagurus longicarpus*) were maintained in running sea water at the Marine Biological Laboratory at Woods Hole, Mass. The

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pagurids fed on fragments of clam muscle which were always available to them. They appeared active and healthy, molting frequently. All of the molts contained apostome ciliates identified by silver impregnation as *Gymnodinioides* sp. and *Hyalophysa* sp. (probably *Gymnodinioides inkystans* and *Hyalophysa chattoni*). Earlier studies have shown that phoronts occur principally upon the gills (Chatton and Lwoff, 1935; Trager, 1957), and according to observations in connection with our experiments, smaller individuals are more heavily infested than larger individuals.

The appendages of premolting *P. longicarpus* appear grayish, changing to a dark slate gray just before molting. Observed in the dissecting microscope the gray color is iridescent. For several days after molting the new exoskeleton appears reddish.

To prepare for *in vitro* experiments on excystation the pagurids were freed from their mollusc shells by shattering the latter with a jack knife handle. The crab was picked from the fragments and deposited in a petri dish half full of sea water. The area just behind the eyes was pierced with sharp forceps, and the crab was thus pinned to the substrate. The carapace was tested with other forceps to see if the old exoskeleton could be lifted from its surface. Then the carapace was ripped off, and the gills were excised, flush with the surface if possible. They floated free in the water until they could be picked up one at a time with two insect pins and deposited in a drop of sea water on a slide. When all the gills were collected, they were covered by a coverslip and examined immediately in the compound microscope. The extent of infestation was determined and any signs of metamorphosis in the phoronts were noted. Metamorphosis can be recognized by the crowding of food reserves to one side of the organism, the finely granular appearance of the cytoplasm, and the mid-ventral rather than posterior position of the contractile vacuole (Bradbury and Trager, 1967). If molting had progressed so that excysted trophonts were present on the slide, the gills from this crab were discarded. Under the higher magnifications of the compound microscope we noted that freshly excysted trophonts seemed attracted to shreds of flesh attached to gills. While swimming near these shreds of flesh, they became somewhat enlarged and had bright red food vacuoles in their interior.

One to four depression slides were placed in sterile petri dishes on moistened filter paper. All the solutions tested and their controls contained 500 units penicillin and 0.05 mg. streptomycin per ml. Various concentrations of glycogen or of glucose in sea water were put in the concavities of the depression slides, and one or two gills were transferred to each depression. These preparations were examined at intervals with a dissecting microscope for the appearance of moving trophonts. In cases where organisms excysted but did not feed, identification was confirmed with the compound microscope.

In experiments testing blood from alien genera of crabs for stimulation of excystation of phoronts on *Pagurus* gills, blood was taken by hypodermic needle from the articulation between the body and an appendage and mixed in equal volume with a sea-water-antibiotic mixture. Donor crabs were *Carcinus maenas* and *Uca pugilator*. Both green and orange *C. maenas* were maintained in the laboratory. The green form was thought to be far from its next molt. The orange form was believed to be close to molting, although subsequently more than a month passed before any animals molted in the laboratory. *U. pugilator* was considered

TABLE I

Effect of glycogen on experimental excystation of apostome phoronts

"Gray" *Pagurus longicarpus*	Solution	Number of trophonts* after (hours)			
		3	6	8–10	17–20
A†	Sea water		1	1	
	0.25% glycogen		1	1	
	0.12% glycogen		4	4	
	0.05% glycogen		4	6	
B	Sea water	0	0	0	
	0.25% glycogen	0	7	13	
	0.12% glycogen	3	1	1	
	0.05% glycogen	0	0	4	
C	Sea water		0	0	1
	0.25% glycogen		0	0	2
	0.12% glycogen		0	0	1
	0.05% glycogen		0	0	0
D†	Sea water	1		0	
	0.25% glycogen	4		0	
	0.12% glycogen	0		0	
	0.05% glycogen	7		20	
E	Sea water	0		0	1‡
	0.25% glycogen	0		0	0
	0.12% glycogen	0		0	0
	0.05% glycogen	0		0	0

* Blank space indicates no observations made.

† Crabs A, B, C were used in one experiment; Crabs D and E in a later one.

‡ After 20 hours the phoronts remaining on the gills were counted and examined for signs of metamorphosis. Crab D gills in 0.25% glycogen had 27 phoronts, some of which showed metamorphosis; in 0.12% glycogen there were 87 phoronts, many showing metamorphosis; in 0.05% glycogen there 19 phoronts, many showing metamorphosis; in the sea water control were 45 phoronts, none showing metamorphosis. Gills from Crab E in 0.25% glycogen had 12 phoronts, from 0.12% glycogen, 9 phoronts, from 0.05% glycogen 19 phoronts, and in the control 28 phoronts. (No phoront from Crab E showed metamorphosis.)

premolt because of the appearance of an intense blue pigmented patch medially and anteriorly on its carapace.

As a control for the blood and glycogen experiments, gills from each crab were put into separate depressions containing the sea-water-antibiotic mixture.

In some cases Chatton-Lwoff stains were made of gills before the experiment (Corliss, 1953) to see whether any metamorphosis had occurred. In other cases gills at the end of the experiment were stained to see if metamorphosis had begun. Preparing material for staining was so time-consuming that it was not feasible to make Chatton-Lwoff stains a routine procedure in experiments.

RESULTS

Excystation stimulated by blood of other genera of crab

Trager (1957) has shown that *Gymnodinioides* on *Pagurus longicarpus* near molting will excyst more readily in its host's blood than in the blood of *P. longi-*

Table II

Effect of blood and glycogen on time of excystation of phoronts*

Solutions	Number of trophonts† after (hours)			
	3	6	9	20
Sea water	0	0	1	30–40
1.0% glycogen	5	5		0
0.5% glycogen	1	1		4
0.25% glycogen	4	8		30–40
0.12% glycogen	4	10		30–40
Blood from orange *Carcinus maenas*				
16%	4	9		30–40
4%	0	3		30–40
2%	0	2		30–40
Blood from green *Carcinus maenas*				
16%	0	0		0
4%	0	0		0
2%	0	0		0

* Solutions were tested on gills from a single gray *Pagurus longicarpus* bearing phoronts in the process of metamorphosis as determined by Chatton-Lwoff silver impregnation. This crab was unusually heavily infested.

† Blank space indicates no observations made.

carpus not near molting. Blood from genera other than the host also seems effective (Trager and Siddiqui, 1963, unpublished observations). In five experiments phoronts from gills of gray *Pagurus longicarpus* excysted in the blood of *Carcinus maenas* and in one case in the blood of *Uca pugilator*.

In the first experiment two engorged trophonts appeared within 11 hours in blood from a green *Carcinus maenas*. *Uca* blood had no effect, and phoronts from the gills of a red *P. longicarpus* tested at the same time were unaffected by blood from either genus.

Experiment 2: Two engorged trophonts appeared within four hours in blood from a green *Carcinus maenas,* but gills from the same crab were not affected by blood from an orange *C. maenas* nor did excystation occur in the sea-water control.

Experiment 3: A single trophont appeared in nine hours in *Uca pugilator* blood from a crab judged near molting. Within 24 hours two other trophonts had appeared. None appeared in the sea-water control.

Experiment 4: Gills from two gray *Pagurus longicarpus* were tested. Chatton-Lwoff silver impregnations of phoronts on Crab A showed no metamorphosis, but many phoronts on Crab B showed metamorphosis, indicating that this crab was probably near molting. Within five hours four trophonts from Crab B gills appeared in the sea-water control. An hour later four trophonts from the gills of the same crab appeared in the blood from an orange *Carcinus maenas*. One trophont from Crab A appeared in blood from an orange *C. maenas*. After 17 hours two trophonts from Crab A appeared in orange *C. maenas* blood and five trophonts from Crab B also appeared in the same blood. Ten trophonts were seen in Crab B's sea-water control.

Experiment 5: Gills from two gray *Pagurus longicarpus* were tested. Silver

TABLE III

Experimental excystation of phoronts from "red" Pagurus longicarpus

"Red" Crab	Concentration	Number of trophonts* after (hours)		
		26–28	40	46
A†	Sea water	0	0	
	0.25% glycogen	2	2	
	0.08% glycogen	0	1	
	0.25% glucose	0	0	
	0.08% glucose	0	0	
B	Sea water	0	0	
	0.25% glycogen	0	0	
	0.08% glycogen	0	0	
	0.25% glucose	0	0	
	0.08% glucose	2	2	
Pooled gills from Crabs C, D, E†	Sea water	0		1
	0.50% glycogen	14		2
	0.25% glycogen	9		10

* Blank space indicates no observation made.

† Crabs A and B were used in one experiment; Crabs C, D, E in a later one.

impregnation of the gills of Crab B showed metamorphosis in the phoronts. In less than four hours one trophont from Crab B gills appeared in blood from a green *Carcinus maenus* and five trophonts in blood from orange *C. maenas*. In 14 hours three trophonts from Crab B gills appeared in blood from green *C. maenas* and six trophonts in blood from an orange crab. Eleven trophonts from Crab B were not present in the sea-water control. A single trophont from Crab A gills appeared in blood from a green *C. maenas* and another trophont appeared in the sea-water control.

In the last two experiments the drawbacks to using relatively high concentrations of blood were especially obvious. The blood mixtures were viscous and cloudy, and the gills were blackened.

It should be noted that excystation of phoronts which had already metamorphosed occurred also in the sea-water controls but generally later than in the blood mixtures.

Effects of glycogen on metamorphosis and excystation

In preliminary experiments gills from grey *Pagurus longicarpus* were placed in 1.0, 0.5, 0.25 and 0.12% concentrations of glycogen and in sea water alone (as always with antibiotics). Within 4 hours trophonts had appeared in 0.12% glycogen only. The results of further experiments with gills from grey *Pagurus* are summarized in Tables I and II. With the exception of Crab E (Table I), where only one trophont appeared altogether, trophonts regularly appeared earlier and in larger numbers in the presence of glycogen. As in the earlier experiments, blood from an orange *Carcinus maenas* was effective, but that from a green one appeared to be inhibitory (Table II).

Especially significant were the results obtained when glycogen was used with phoronts on gills from post-molt "red" *Pagurus longicarpus.* Whereas excystation had never been observed in such material even when placed in homologous or heterologous blood, and despite the relatively light infestation of such crabs, appreciable numbers of trophonts appeared in the presence of glycogen (and in one case of glucose) (Table III). None of these trophonts fed. They swam about actively for various periods of time and then degenerated and died.

DISCUSSION

The change from phoront to trophont involves two steps, first metamorphosis within the cyst and then excystation. Both of these evidently can be induced *in vitro* by appropriate concentrations of crab blood or of glycogen. It is worthy of note that three different commercial preparations of highly purified glycogen all gave the same results. Of special interest is the fact that a glycogen-sea water mixture induced metamorphosis and excystation of *Hyalophysa* and *Gymnodinioides* in gills of recently molted "red" *Pagurus.* Certainly glycogen must be one of the factors involved in the change from phoront to trophont occurring at the time of molting of the host crab. That it is the only factor seems unlikely, since the numbers of trophonts obtained *in vitro* rarely approached those observed in shed skins under natural conditions. Other obvious factors which might play a role are changes in oxygen tension (this may decrease in the gill chamber shortly before molting) and pH (exuvial fluids are strongly alkaline with a pH around 9). At present we can only speculate as to how a substance like glycogen can have a morphogenetic effect involving extensive rearrangement of previously formed organelles (Bradbury and Pitelka, 1965; Bradbury, 1966), as well as new synthesis.

The occasional excystation which occurred in the sea-water controls from phoronts already metamorphosed, and the still rarer and delayed instances of metamorphosis in the controls, may have resulted from substances exuding from the gill tissue. This might be comparable to Miyashita's (1933) observation of excystation of *Hyalospira* occurring when the cysts were put under a coverslip with fluid squeezed from the host shrimp.

The histotrophic apostomes, which excyst when the host is injured, and those of the genus *Foettingeria* which excyst when the host is eaten (usually by a coelenterate) differ from the exuviotrophic apostomes, dealt with in the present work, in that they undergo step 1, the metamorphosis, almost immediately after encysting on their host. Hence they are ready to excyst, as it were, at a moment's notice, an obvious requirement if excystation is to occur in response to instantaneous events such as injury or predation. The exuviotrophs on the other hand do not undergo their metamorphosis until immediately before their host will molt, in response to altered physiological conditions in the host. One of these effective conditions has now been shown to involve an increased concentration of glycogen.

SUMMARY

To test the hypothesis that a substance or substances normally present in the blood and tissues of pre-molt crabs initiates metamorphosis and subsequent excystation of apostome phoronts, we put excised hermit crab gills bearing *Hyalophysa* and *Gymnodinioides* phoronts in sea-water-antibiotic solutions containing blood

from other species of crabs or low concentrations of glycogen. As a control gills from the same crab were put into the sea-water-antibiotic mixture alone. The glycogen solutions were more effective in inducing both metamorphosis and excystation of the phoronts than the heterologous crab blood. In repeated trials, in which the controls showed no changes, metamorphosis and excystation occurred with glycogen concentrations of 0.12 to 0.5%. This was true even if the phoronts were on gills taken from a hermit crab only recently molted and hence far from its next molt.

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AN AUTORADIOGRAPHIC STUDY OF THE UTILIZATION OF FREE EXOGENOUS AMINO ACIDS BY STARFISHES[1]

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In a previous report (Ferguson, 1967) evidence was provided that at least two very different species of starfishes are capable of making extensive use of dissolved amino acids and glucose which might occur in their environment. That paper confirmed results of earlier experiments performed by Stephens and Schinske (1961), demonstrating that these two starfishes, along with many other types of invertebrates, can remove glycine from their surrounding medium. In addition, it showed that in most cases the dissolved nutrients taken up by these forms are retained almost exclusively in body wall components and do not appear to be translocated into the internal organs.

As that study was based on quantitative measurements of labeled material taken up into the different major regions of the body, it could not reveal the precise cellular location of uptake, or possible translocations of the absorbed nutrients within the regions studied. However, it was inferred, largely on the basis of previous autoradiographic observations of animals fed clams containing labeled nutrients (Ferguson, 1963a, 1963b), that much of the epidermis was involved in the absorptive process.

The present investigation was undertaken in order to locate more precisely the significant areas in which the exogenous nutrients are taken up, and to determine the normal limits of distribution of these nutrients within the body. The observations have been limited to the localization of absorbed amino acids, since these compounds do not appear to be ingested to any significant extent under the experimental conditions that have been employed. As shown previously (Ferguson, 1967), dissolved sugars are sometimes taken up orally. For that and other reasons their uptake presents a considerably more complex picture, which will require further analysis.

MATERIALS AND METHODS

The starfishes used in these experiments were freshly collected specimens of *Asterias forbesi* and *Henricia sanguinolenta,* both from the Woods Hole region, and *Echinaster spinulosus*[2] from Tampa Bay, Florida. Only individuals about 3

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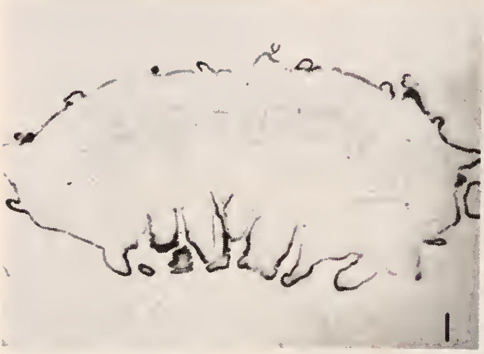
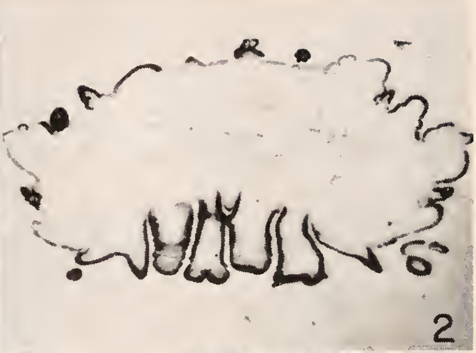
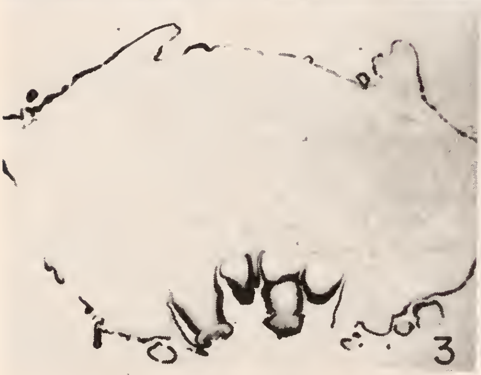
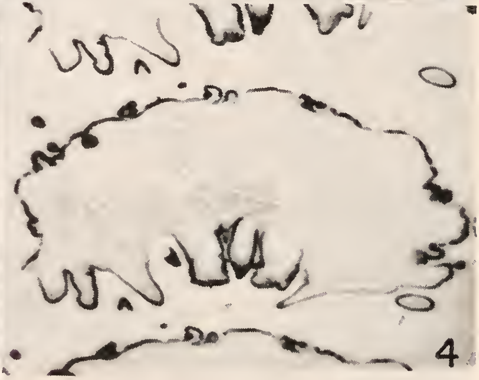
[1] Supported by NSF grants GB 2209 and GB 4994.

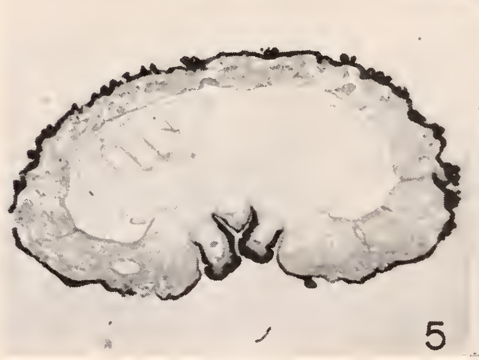
[2] After surveying the available literature and consulting with the curators of the echinoderm collection of the Marine Laboratory of the Florida Department of Conservation (who have been in communication with the U. S. National Museum on the problem), it is evident that the taxonomy of the echinasters is at the present time in a state of considerable confusion. The forms used here were all obtained from an apparently homogeneous population located on tidal grass flats near the east side of the southern end of the Sunshine Skyway bridge.











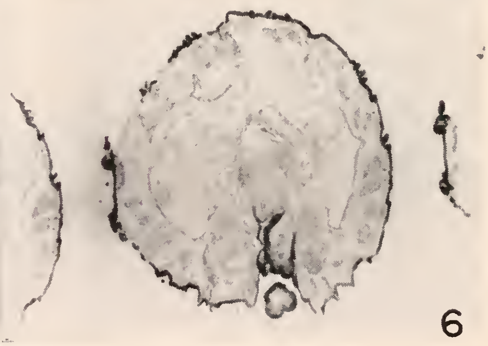


FIGURE 1. Autoradiograph of a section through the mid-region of a ray of a specimen of *Asterias* sacrificed 1 hour after being placed in C^{14}-labeled amino acid medium. Darkened epidermis indicates uptake of the labeled amino acids into that region. No significant radioactivity is detectable in other areas. This preparation, like those shown in all of the following figures, was unstained. 12 ×.

FIGURE 2. Autoradiograph of *Asterias* ray similar to Figure 1, but this specimen was sacrificed after 8 hours in the medium. While incorporation of the labeled amino acids has been somewhat greater, the radioactive areas are essentially the same as those in Figure 1. 12 ×.

FIGURE 3. Equivalent autoradiograph of a sectioned ray of *Asterias* incubated 8 hours in the labeled amino acid medium and then placed in circulating sea water for 3 days before sacrifice. The tracer has not moved from the epidermal areas in which it was initially taken up. 12 ×.

to 5 cm. in diameter were selected for study. For the Woods Hole species, each animal was placed in 50 ml. of filtered sea water to which had been added 0.5 microcurie (0.0033 mg.) of a mixture of 15 uniformly labeled L-amino acids (representing a synthetic algal protein hydrolysate mixture, manufactured by the New England Nuclear Corp. of Boston, Mass.). One group of specimens was left in the medium for 1 hour and then rinsed in sea water and sacrificed. The remaining animals were left in the medium for 8 hours before being placed in fresh sea water. Of these latter specimens, some were sacrificed immediately while other groups were placed for 3 and 20 days in circulating sea water before being sacrificed. The last two groups were allowed to feed *ad libitum* on small clams during their periods of retention.

In the case of the specimens of *Echinaster*, 6 individuals were placed in 100 ml. of filtered sea water containing approximately 4.0 microcuries of uniformly labeled glycine-C^{14} in a 0.1 m*M* solution. Some individuals were removed from this medium and sacrificed after 1, 6, and 24 hours. The specimens remaining after 24 hours were rinsed and placed in tanks of uncontaminated sea water for periods of 3, 9, and 20 days.

Sacrifice was accomplished by severing the rays from the disk and cutting each of them transversely into equal portions. The pieces obtained were then fixed for 3 days in Bouin's solution diluted 50% with water (to reduce the rate of decalcification). They were then dehydrated through alcohols, cleared in xylene, and embedded in paraffin in a vacuum oven. Sections of the material were cut at 10 microns thickness, mounted on "subbed" slides, deparaffinized, rehydrated, and covered with AR-10 stripping film as recommended in the directions for use of the product as supplied by the Eastman Kodak Co. After air-drying, the slides were kept in black plastic boxes in a refrigerator for up to 6 months and then developed 20 minutes in Kodak D-19 developer. After fixing, the preparations were rinsed, dehydrated in alcohol, cleared in xylene, and mounted with a coverslip, using a diluted synthetic medium. In most cases, staining was not employed.

This procedure, of course, retains and makes visible only the labeled amino acids which have become firmly bound up in the tissues. As was previously shown (Ferguson, 1967), this actually represents a rather sizable proportion of the total amino acid taken up by the forms. In the case of *Asterias*, for example, the bound fraction was found to range from approximately 65% in 1-hour specimens to 90% in those kept 20 days.

RESULTS

Darkened areas of the emulsion of the stripping film covering the sections demonstrated that all the animals had taken up and retained significant amounts of the labeled amino acids which were provided in their medium. The proportion of the amino acids taken up cannot be stated with certainty as the method is not quantitative. The counting of a few aliquots of medium before and after the

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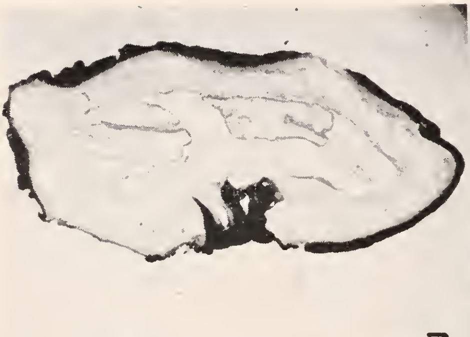
FIGURE 4. Section of *Asterias* ray similar to that in Figure 3, but kept 20 days in circulating sea water. There are only minor differences between the distribution of the tracers in this specimen and the previous ones. These differences stand out more clearly in Figures 13–15. 12 ×.

FIGURES 5 AND 6. Preparations similar to those in Figures 3 and 4, but of *Henricia*. Results are much the same except that the tissues show up more distinctly because of their natural coloration. Both 12 ×.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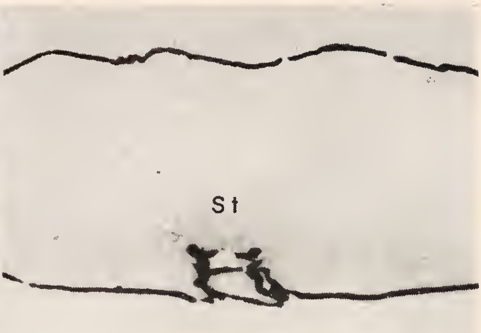

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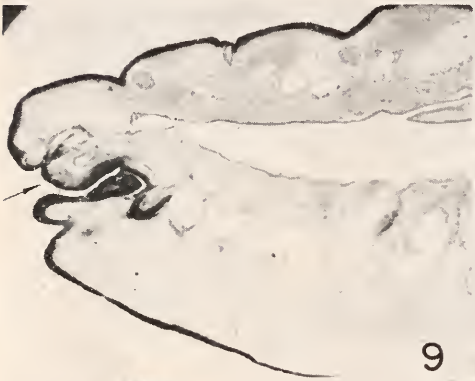
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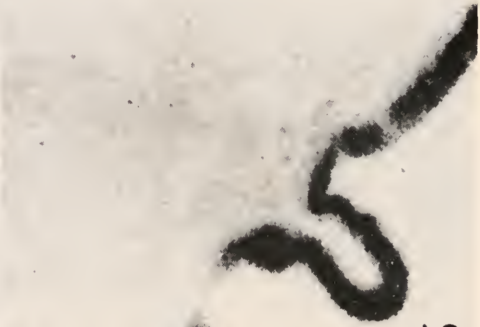
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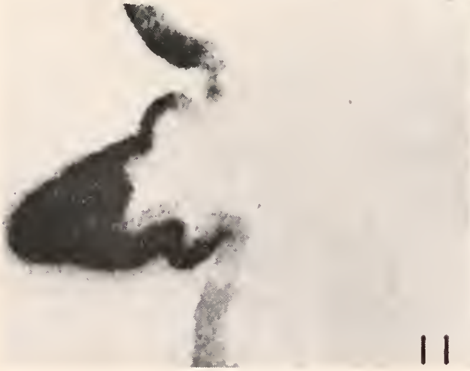
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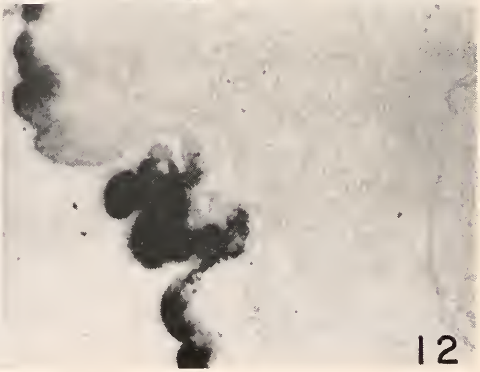
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FIGURE 7. Autoradiograph of specimen of *Echinaster* placed for 24 hours in a medium containing glycine-C^{14}, and then kept in sea water for 20 days before sacrifice. As with the other species, the labeled amino acid has been incorporated into the epidermal regions. Other tissues stand out due to their natural coloration. Greater detail of the ambulacral area is shown in Figure 17. 14 ×.

FIGURE 8. Autoradiograph of transverse section through disk of a specimen of *Echinaster* sacrificed 6 hours after being placed in glycine-C^{14} medium. While the epidermis has incorporated a great quantity of the labeled glycine, apparently none of it has entered the mouth and been taken up by the stomach or other tissues. (St, area of stomach tissue.) 8 ×.

FIGURE 9. Autoradiograph of a longitudinal section of a ray tip of a specimen of *Echinaster* sacrificed 24 hours after being placed in medium containing glycine-C^{14}. Arrow indicates optic

experiments, however, indicated that anywhere from about 30 to 60% of the tracer was removed by the different animals. Doubtless, size and species differences were the main factors involved in the variation.

General observations

In each of the 3 species, the labeled amino acids were taken up and retained primarily by the epidermal cells forming the body surface. This can be seen most clearly in Figures 1–9, which are low-magnification views of typical sections. The greatest uptake appeared to occur in the tube feet, papulae, and some of the spines, but apparently all external areas were involved to some extent. Several workers, including Gislén (1924) and Budington (1942), have described ciliary currents maintained over the surfaces of starfishes, and very likely the intensity of these currents, as well as the degree of exposure of the structures, were significant in determining the quantity of the amino acids taken up by the different regions.

While carbon-14 is considered a low-energy β-particle emitter, the energy of the particles is nevertheless too great to permit precise intracellular localizations. The resolution in the present experiments was probably on the order of about 5 microns. Even at this resolution, it could be observed that the amino acids were rapidly distributed throughout the cytoplasm of the elongated epidermal cells. This fact is illustrated in Figure 10, which shows a portion of a specimen of *Henricia* after a single hour of exposure. The radioactivity is apparently no greater in the external border of the cells than in the basal regions.

In this same figure, as well as in Figures 11 and 12, depicting equivalent areas of specimens of *Asterias* at 8 hours and 20 days, respectively, it can be seen that practically all of the epidermal cells participated in the uptake process. A few lighter regions in the layer, representing areas of less activity, may be observed, however. The significance of these is not certain, but since they generally appear to merge gradually with denser areas, they probably are portions of the epidermis which were more protected from the ciliary currents. Smaller, more distinct areas of lightness, as in the upper portion of Figure 10, can usually be traced in adjacent sections to the larger patches.

Close observation of the epidermis in many cases revealed slight irregularity in uptake of amino acids by the various types of cells making up the layer. The limitations of resolution made it difficult to correlate this unevenness with the different cells in the layer, but after many observations it seems probable that glandular cells tended to accumulate more radioactivity than other types. These differences were relative, since the actual amount of amino acid taken up by any cell chiefly depended on the region in which it was located.

In addition to these variations in the intensity of the label incorporated into

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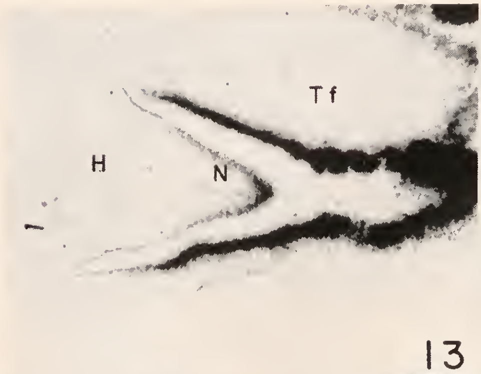
cushion, which took up an appreciable quantity of the labeled amino acid, as did the remainder of the epidermis. The optic cups stand out as small light areas in the optic cushion, because they incorporated little of the tracer. 30 ×.

FIGURE 10. Autoradiograph of a typical section through a papula. *Henricia* after 1 hour of incubation with the labeled amino acids. 90 ×.

FIGURE 11. Preparation similar to that in Figure 10. *Asterias* after 8 hours of incubation with the labeled amino acids. 125 ×.

FIGURE 12. Similar to Figure 11, but specimen kept 20 days after exposure to the labeled amino acids. Note that the radioactivity remains confined exclusively in the epidermis. 85 ×.



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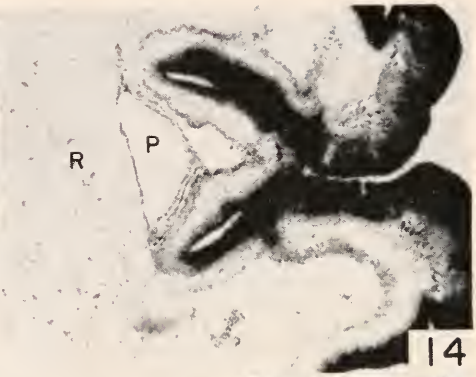

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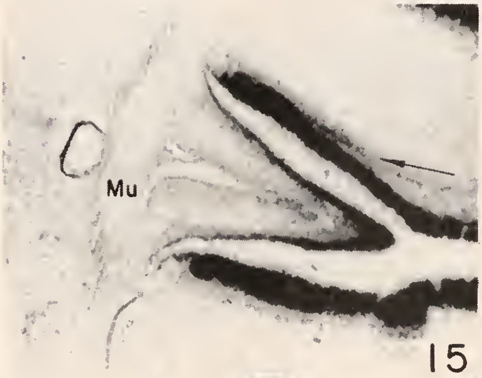

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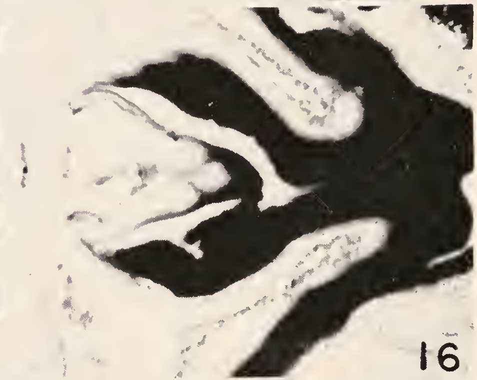

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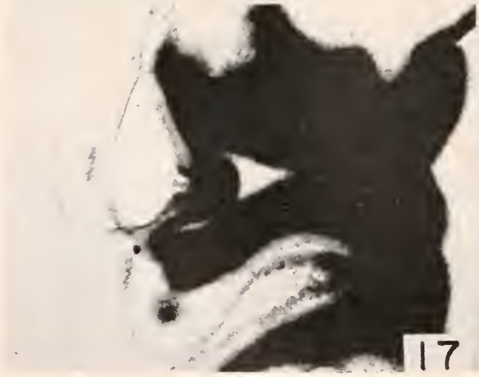

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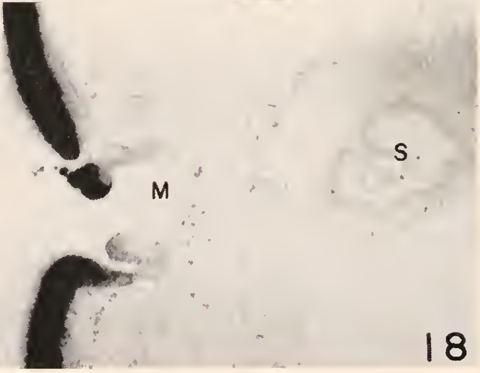

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FIGURE 13. Autoradiograph similar to that in Figure 1 (*Asterias,* 1 hour). Radioactivity is found in epidermis of tube feet and cellular area of radial nerve cord. (H, location of hemal septum; N, radial nerve cord; Tf, tube foot.) 125 ×.

FIGURE 14. Autoradiograph similar to Figure 3 (*Asterias,* 3 days). Small amounts of radioactivity are found in deeper layers of radial nerve cord and tube feet. (R, radial canal of water vascular system; P, perihemal sinus.) 85 ×.

FIGURE 15. Autoradiograph similar to Figure 4 (*Asterias,* 20 days). Some radioactivity is found in deeper layers of radial nerve cord, and in inner layer of tube foot (arrow). Darkening of radial canal is due to artifact of fixation. (Mu, lower transverse ambulacral muscle.) 125 ×.

the surface tissues, gaps may be seen in the epidermal layers shown in some of the figures. These are artifacts caused by various types of damage suffered by the rather delicate layer during preparation of the sections. Also, chemical reduction of the photographic emulsion could be detected in a number of instances. It most commonly occurred over osteocytes, some amoebocytes, and various scattered cells in the digestive system. Such areas could easily be recognized from the fact that they were as common in the 1-hour specimens as those kept 20 days, and the distribution of the silver grains was somewhat different from those produced by radiation.

Observations on specific areas

A number of areas in the different preparations were selected for more detailed study. In general, the observations revealed very little significant variation among the three species in the reaction of these regions to the presence of the labeled amino acids in the medium.

Mouth and stomach. These regions are most clearly shown in Figure 8, which represents a section of a specimen of *Echinaster* after 6 hours' exposure to the glycine-C^{14}. In addition, stomach regions of specimens of *Asterias* sacrificed 3 and 20 days after exposure to the labeled amino acid mixture may be seen in Figures 19 and 21. In all cases, the epidermis extending to and covering the buccal membrane readily took up the amino acid, but practically none of the labeled material worked its way through the esophagus into the stomach. No significant amounts of radioactivity were detected in any portion of the digestive system.

Ray tip. The epidermis on the tips of the rays took up at least as much of the labeled amino acids as did the epidermis of other regions. As can be seen in the specimen of *Echinaster* (exposed 24 hours) shown in Figure 9, the terminal tentacle and the region of the optic cushion were particularly radioactive. The optic cups, which apparently were deficient in cytoplasmic material (Smith, 1937), stand out as lighter areas in the optic cushion.

Ambulacral groove. Because of the presence of many important structures, the region of the ambulacral groove was closely scrutinized in all the specimens. Typical results are shown in Figures 13–17. The amino acids were very readily taken into the epidermal layers of both the sides and sucker portion of the tube feet. Considerable activity was also found in the radial nerve cord, mostly localized in the basal cellular layer, but present to some degree in the fibrous area. In addition, small but possibly significant traces of radioactivity could be discerned in adjacent tissues, especially in specimens kept for the longer periods of time. Such activity

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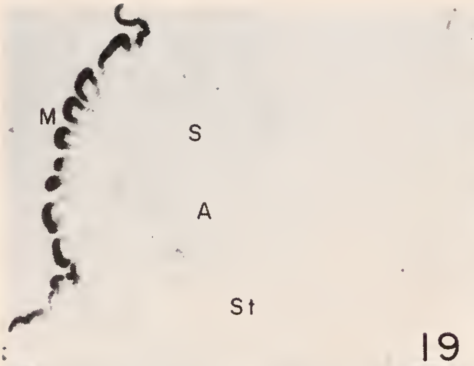
FIGURE 16. Preparation similar to those in Figures 13, 14 and 15, but of *Echinaster* after 24 hours of exposure to glycine-C^{14}. 85 ×.

FIGURE 17. Specimen of *Echinaster* similar to that in Figure 16, but kept additional 20 days in sea water. Note that a small portion of the large amount of glycine-C^{14} taken up by this animal has found its way into the deeper areas of the nerve cord and the tube feet. A clump of radioactive cells (amoebocytes?) in apical end of lower tube foot are especially conspicuous. 85 ×.

FIGURE 18. Autoradiograph of a transverse section through edge of the madreporite and stone canal of a specimen of *Echinaster* after 6 hours exposure to glycine-C^{14}. None of the labeled glycine has penetrated into the water vascular cavity. (M, madreporite; S, location of stone canal.) 85 ×.



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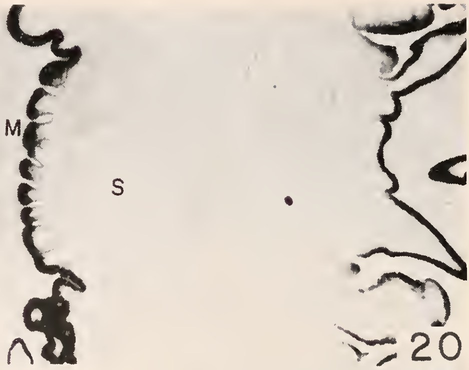

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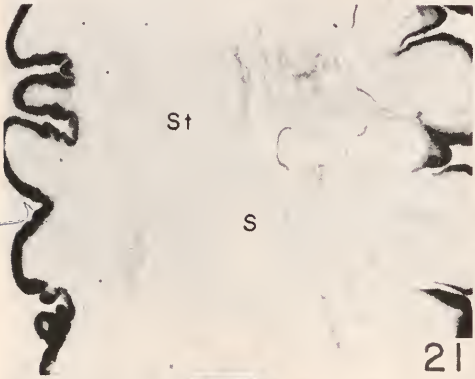

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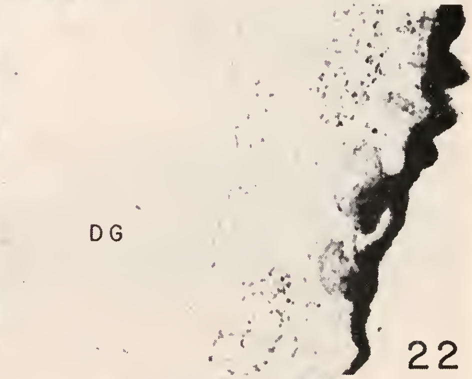

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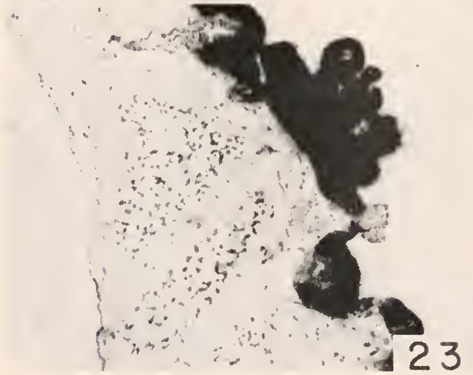

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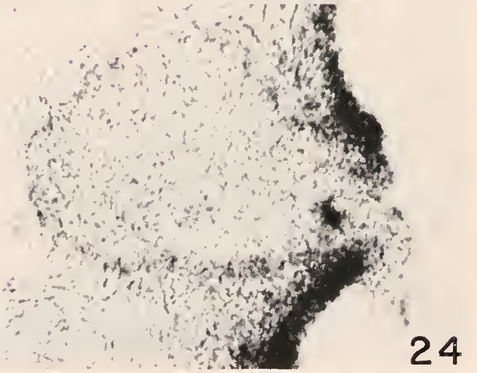

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FIGURE 19. Autoradiograph of a transverse section through the madreporite region of a specimen of *Asterias* 3 days after exposure to the labeled amino acids. Considerable uptake has taken place in the outer epidermal areas of the madreporite, but no significant radioactivity is evident elsewhere. (M, madreporite; S, location of stone canal; A, location of axial gland; St, area of stomach tissue.) 33 ×.

FIGURE 20. Similar to Figure 19, but this specimen was kept 20 days. (S, location of stone canal.) 30 ×.

FIGURE 21. Different region of the same specimen as in Figure 20. (St, area of stomach tissue; S, location of stone canal.) 30 ×.

FIGURE 22. Autoradiograph of a transverse section through a ray of a specimen of *Henricia* after 8 hours exposure to labeled amino acids. Large epidermal glands (right) may be seen

was seen in Lange's nerve (the motor neural complex), the hemal septum, and the aboral lining of the perihemal canal. Only background reduction of the emulsion could be detected in the various muscles, connective tissue, and water vascular canals in this region. The lateral motor centers described by Smith (1950), located in the epidermis juxtalateral to the bases of the outer rows of tube feet, took up relatively little amino acid, possibly because of their deep, sheltered position.

Madreporite, stone canal, and axial gland. While not without controversy (*cf.* Nichols, 1966), it is probable that the madreporite and stone canal serve as structures which bring sea water into the water vascular system for ultimate use in the protraction of the tube feet and, by pressure-filtration through the ampullae, for replenishment of the coelomic fluid. If sea water does enter these structures, they could possibly also be significant routes for the uptake of exogenous dissolved nutrients and the distribution of organic materials to the interior regions of the oral body wall. Figures 18–21 illustrate the observations made on these structures.

In every case the radioactivity was confined to the most exposed regions of the madreporite epidermis. No significant activity could be observed in sections through any level of the stone canal, the axial gland, or the tissues lining the perihemal sinus surrounding the axial gland. At least three different possibilities could explain these results. First, there may have been no flow of medium through the structures. Even though the animals were confined to small containers during their exposure to the amino acids, this possibility seems unlikely since the tube feet generally remained quite active and presumably created at least some demand on the water vascular channels for water. Second, the internal tissues may not have possessed an affinity for the labeled amino acids. In no previous study, however, have starfish cells failed to exhibit uptake of these materials. Third, the amino acids could have been completely removed from the inflowing water by the superficial tissues of the madreporite. This final hypothesis appears to be the most reasonable.

Large epidermal glands. The possible significance of small glandular cells in producing irregularities in the intensity of radioactivity in the epidermis has already been mentioned. In *Henricia* and *Echinaster* there are additional very large glands which extend deep into the connective tissue of the body wall. These apparently produce a copious, mucus-like secretion. These glands are of special interest as they must present a significant demand for nutrients in an area somewhat distantly removed from the presumed major source of supply, *i.e.*, the coelomic fluid (*cf.* Ferguson, 1964a, 1964b).

The large epidermal glands could readily be recognized in the sections (Figs. 22–24). In a number of the specimens incubated for the shorter periods of time, the tracer was seen to have penetrated at least part way down into the glands (Fig. 22). In the specimens maintained for a number of days after they were

extending down into body wall connective tissue, and radioactivity has penetrated into the glands. (DG, location of digestive gland.) 85 ×.

FIGURE 23. Similar to Figure 22, but specimen kept additional 3 days in circulating sea water. Contents of large epidermal gland is seen being extruded. Some radioactivity is found in this material. 85 ×.

FIGURE 24. Large epidermal gland in a specimen similar to that in Figure 7 (*Echinaster*, 21 days). Most of the radioactivity has left the gland, which is seen filled with refractile particles. 360 ×.

removed from the labeled medium, however, only small quantities of the radioactive material could be detected in these structures. Little is known of the chemical nature of the secretion of the glands. Hyman (1955) refers to it simply as "gelatinous." The present observations, while admittedly inconclusive, do suggest that at least part of the material is derived from external amino acids sources.

Translocation of epidermally absorbed nutrients

Translocation of the epidermally absorbed amino acids from the epidermal cells to other regions appears to be very limited. This observation is illustrated in Figures 1–4, representing comparable transverse sections through the medial portion of the rays of *Asterias* specimens sacrificed after different periods of time. The distribution of radioactivity in the 20-day specimen (Fig. 4) is almost identical to that in the 1-hour specimen (Fig. 1). In none of the preparations can significant amounts of the tracers be detected in the digestive glands, ampullae, ossicles, connective tissue, and most other parts. The same observation holds true for the two transverse sections of *Henricia* (Figs. 5 and 6), representing 3- and 20-day specimens. Natural coloration in these animals makes their tissues stand out more clearly in the photographs. A 21-day specimen of *Echinaster* may be seen in Figure 7, and, again, nearly all of the tracer remained localized in the epidermis. Figures 10–12 are more highly magnified views and clearly illustrate the sharp demarcation, even after 20 days, between the epidermal layers retaining the labeled material and the underlying connective tissue in which is found only normal background activity.

A few cases were seen, however, which could possibly be interpreted as slight degrees of translocation. A gradual increase in radioactivity of some of the oral perihemal areas has already been mentioned. In addition, a small inward movement of the tracer could also be detected in many of the more heavily exposed tube feet (Figs. 15 and 17). Most of this activity became localized in the peritoneal cells lining the lumina of these structures or in a clump of cells, possibly amoebocytes, usually found in the lower apical ends of the cavities. In any case, the labeled nutrient finding its way into these places probably could not represent more than a small fraction of a per cent of the total amino acid taken up by the adjacent epidermis.

DISCUSSION

The results of the foregoing experiments seem to illustrate a considerable dependence of the starfish epidermal cells on soluble amino acid sources in the external environment. There are no areas of the body surface that do not appear to possess this property. While some variation in uptake was noted among the different cell types which make up the epidermis, and among the different body regions, large quantities of the labeled amino acids were taken up everywhere the medium came in contact with the animals.

Relatively little is known about the mechanism by which these substances are absorbed into the cells. The autoradiographs produced no indication of significant uptake of the compounds on the cell surfaces, but rather, revealed that the substances were taken in rapidly (at least in less than an hour) through the cell membranes and evenly distributed throughout the cytoplasm of the cells. Furthermore, as only insoluble materials are visualized by the technique, much of the absorbed amino acid must have also been converted, with equal rapidity, into polypeptides or other compounds. It is possible that this conversion is the driving force of a "facilitated diffusion" of the compounds into the cells, and that no other "active" mechanisms are necessary for transport. Such an hypothesis, however, is at the present time rather difficult to defend, since it may be calculated from previous results (Ferguson, 1967) that a suitable concentration gradient for entry would not appear to exist.

Possibly further evidence against a diffusion mechanism as a significant factor in the uptake process is provided by the fact that the acellular optic cups stood out as areas of little uptake, while the cellular areas surrounding them demonstrated great affinity for the tracers. Perhaps the optic cups are relatively impermeable to the amino acids, but more likely, they lack the specific absorptive machinery necessary for the maintenance of the process.

The almost universal failure of the absorbed amino acids to move out of the epidermal cells into other regions of the body probably is a point of great functional significance. It might imply that there exists some sort of barrier beneath the epidermal cells that would bar most of the inward migration of the amino acids. Such a barrier, if it existed, presumably would also reduce movement of metabolites from inside the body outward to the epidermal cells, and would leave the epidermal cells to subsist in a fairly autonomous fashion at the surface of the animal. This barrier could have its greatest importance as a mechanism in preventing the loss of nutrient pools maintained in the body fluids.

Close examination of some of the observations reported in this investigation, however, makes the existence of a significant barrier of this kind quite unlikely. First of all, there were a few areas, such as in the tube feet, in which small but distinct quantities of the tracers did appear to migrate inward. Secondly, the inability of the labeled amino acids in the medium to penetrate through the outer regions of the madreporite, buccal membrane, and other areas indicates an extremely strong affinity on the part of the epidermal cells for the uptake and retention of the compounds. This affinity of the epidermal cells for the exogeneous amino acids is so great, it seems likely that they are also capable of drawing on any nutrient pools which might tend to form in the ground substance of the underlying connective tissue. In this view, the epidermal cells attract and absorb amino acids from all directions. Such action causes diffusion gradients to be established in the connective tissue layers, and these gradients are slanted towards the epidermal cells except when overdriven by large quantities of substances taken up from the external medium.

If this hypothesis is correct, it is the epidermis itself which is the primary barrier against both the movement of externally absorbed compounds into the deeper regions of the body, and the loss to the environment of nutrient pools maintained in the body fluids. Actually, it is unlikely that more than very small amounts of nutrients normally reach the epidermis from internal sources. The concentration of amino acids maintained in the coelomic fluid are very low (Ferguson, 1964a) and the diffusion distance, through the connective tissue of the body wall, is relatively great. Even with complete removal of the amino acids by the epidermal cells, the gradient of diffusion toward these cells would be in most cases quite slight. The concentrations of amino acids expected in the immediate micro-environment of the animals generally would be a much more suitable source of supply.

It may be noted further that the modified epidermal tissue forming the lateral motor complexes, which were found to be somewhat protected from the external sources of amino acids, are bordered on their basal side by extensions of the perihemal sinus. These in turn are separated by only a tenuous tissue layer from the visceral coelomic cavity. The lateral nervous centers are thus in a position to receive nutrients *via* an internal route by mechanism previously described (Ferguson, 1964b). As extensions of the perihemal sinus can also be traced to the vicinity of most of the major muscles in the oral side of the body, such as the lower, transverse ambulacral muscle shown in Figure 15, this cavity is probably a most important route for the distribution of endogenous nutrients within that portion of the body.

It is concluded, then, that while starfishes generally appear to make extensive use of exogenous sources of free amino acids, these are of negligible importance to most of the tissues of the body, and in actuality, they only benefit the epidermis and some of the structures immediately derived from it. This benefit, however, appears to be very significant, and probably in many cases represents the chief source of nutrition for these cells. Underlying cellular regions are generally situated in close proximity to various body cavities from which the cells presumably obtain nutrients distributed from endogenous sources.

SUMMARY

1. Specimens of *Asterias forbesi*, *Henricia sanguinolenta*, and *Echinaster spinulosus* were exposed up to 24 hours to sea water media containing C^{14}-labeled amino acids. The distribution of the labeled compounds after periods of 1 hour to 21 days was determined by means of stripping-film autoradiographs of histological sections of the specimens.

2. The results from all three species were very similar. Large quantities of the supplied amino acids were found to have been taken up and retained by the epidermal cells in almost all regions of the body, and especially in those parts most in contact with the external medium. Variations in uptake between the different types of cells making up the epidermal layer appeared to be only minor and relative.

3. The amino acids were found not to have penetrated through the madreporite channels or through the buccal opening. If fluid entered these regions, the amino acids were presumably completely removed by the adjacent superficial cells, which always demonstrated large amounts of uptake.

4. Secretions by the large epidermal glands characteristic of *Henricia* and *Echinaster* appeared to contain at least some material derived from the exogenous amino acids supplied.

5. There was very little evidence of movement of the absorbed amino acid out of the epidermal cells within the 3-week period during which the observations were continued. Only slight translocation occurred in some of the tube feet and in the region of the radial nerve cord.

6. It is concluded that absorption of environmental amino acids (and probably other compounds) by the epidermis is an important and often principal source of

nutrition for the cells making up this tissue; other areas of the body, however, depend almost exclusively on endogenous sources of nutritive substances, probably delivered by the fluids circulating through the various body cavities.

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THE EFFECTS OF ALTERNATING LONG AND SHORT DAILY PHOTOPERIODS ON GONADAL GROWTH AND PITUITARY GONADOTROPINS IN THE WHITE-CROWNED SPARROW, ZONOTRICHIA LEUCOPHRYS GAMBELII[1]

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The photoperiodic induction of the development of gonads in many species of birds of mid and high latitudes has become an extensively investigated phenomenon. (See Farner, 1959, 1961; Farner and Follett, 1966; Wolfson, 1966, for reviews.) An interesting characteristic of the response, at least in some species, is that the stimulatory photoperiod need not consist of an uninterrupted daily period of light; it may instead be replaced effectively by a series of flashes of light following an otherwise nonstimulatory photoperiod, or simply by a daily series of adequately spaced short photoperiods. (See, for example, Benoit, 1936; Burger, Bissonnette, and Doolittle, 1942; Straffe, 1950; Jenner and Engels, 1952; Farner, Mewaldt, and Irving, 1953; Farner, 1958, 1959, 1964b, 1965a; Wolfson, 1959a,b,c,d, 1960, 1966.) Earlier investigations of this characteristic of the photoperiodic testicular response in the White-crowned Sparrow (*Zonotrichia leucophrys gambelii*) led to the hypothesis of a "carry-over period" (Farner, Mewaldt and Irving, 1953; Farner, 1958, 1959, 1964a) that rationalized the induction of growth of the testes in response to flashes of lights by assuming the persistence of a light effect into the ensuing dark period. More recently the interpretation of responses to interrupted light has been complicated by the discovery (Hamner, 1963, 1964, 1965) of a circadian function in the mechanism of photoperiodic testicular response of the House Finch (*Carpodacus mexicanus*). Similar functional relationships have been confirmed for the House Sparrow (*Passer domesticus*) by Menaker (1965), for the Slate-colored Junco (*Junco hyemalis*) and the Bobolink (*Dolichonyx oryzivorus*) by Wolfson (1965a, b, 1966), and for *Z. l. gambelii* by Farner (1964b, 1965b). Farner (1965b) presented evidence that suggests that the nature of this circadian component in *Z. l. gambelii* is a periodicity in photosensitivity of the response mechanism.

These demonstrations of a circadian function in the response mechanism have necessitated modification of the original "carry-over" hypothesis. This modification is based on a summation of derived rates from the photosensitivity curve (Farner, 1965b) for a series of daily photoperiods of increasing duration (8L 16D through 20L 4D to continuous light) and a comparison of the rates obtained by summation with those based on direct measurements of responses to the same series of photo-

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periodic treatments (Farner and Wilson, 1957). The rates obtained by summation were found to exceed the measured rates as a regular function of the duration of the photoperiod. The simplest explanation for this relationship is that the rates presented by Farner (1965b) must include "carry-over effects" from the 2-hour photoperiods and that the magnitude of the "carry-over effect" also is a function of the cycle in photosensitivity.

Although direct evidence is lacking, it appears that the "carry-over" effects from brief flashes (1–10 seconds) are of short duration and are probably associated with the function of receptors or neural transmission (Farner, 1958). Other functions that could provide possible bases for "carry-over effects," especially of longer duration, include the release and transport of neurohormone from the median eminence, release of gonadotropin from the adenohypophysis, the survival time of gonadotropin in the circulating blood, and the nature of the action of gonadotropin on the growth processes of the gonad. There is now experimental evidence for the existence of "carry-over effects" of long duration (hours or days), that may be explainable on the basis of one of these mechanisms. For example, in *Carpodacus mexicanus* a stimulatory photoperiod has a growth-promoting effect on the testes for as long as 72 hours after the cessation of the photoperiod (Hamner, 1964). The experiments described herein were designed to investigate further these long-persisting "carry-over effects" in *Zonotrichia leucophrys gambelii* by subjecting birds to a variety of cyclic photoperiodic treatments. It is the function of this paper to communicate the results of these experiments with respect to the rate of development of the gonads and the concentration of gonadotropin in the adenohypophysis.

THE EXPERIMENTS

Basic to the design of the experiments are certain characteristics of photoperiodically induced gonadal growth in *Zonotrichia leucophrys gambelii* (Farner and Wilson, 1957; Farner, 1959, 1964b; Farner *et al.*, 1966). Of primary importance is the logarithmic nature of testicular growth from resting weight (*ca.* 2 mg.) to *ca.* 250 mg. and of ovarian growth from resting weight (*ca.* 5 mg.) to *ca.* 50 mg. under photoperiodic stimulation. The relationship between gonadal weight and time subjected to fixed daily stimulatory photoperiods may be expressed quite accurately by:

$$\log W_t = \log W_0 + kt$$

where W_0 is the resting gonadal weight (in mg.), W_t is the gonadal weight on day t after the beginning of photoperiodic stimulation, t is time (in days), and k is the logarithmic growth-rate constant (in days^{-1}).

For these experiments we adopted initially the admittedly oversimplified working hypothesis that each long day causes a fixed logarithmic increment of gonadal growth even though isolated by intervening days with short, non-stimulatory photoperiods. It was assumed that the short, non-stimulatory photoperiods make no positive contribution to gonadal growth, a reasonable assumption since males show no demonstrable growth after many months on 8-hour daily photoperiods (Farner and Wilson, 1957). Although there is a small non-photoperiodic growth of the ovary under such conditions (Farner *et al.*, 1966), it is negligible within the context of these experiments. Thus groups of birds were subjected to a variety of cycles of the type L nS (L = day with long, stimulatory photoperiod; S = day with short, non-stimulatory photoperiod; n refers to the number of short days interposed between long, stimulatory days; it may take the value of 0 or any integer).

If the working hypothesis were correct, it follows that k expressed in cycles $^{-1}$ (rather than days $^{-1}$), should be constant for all groups. A value of k (in cycles $^{-1}$) greater than that obtained with daily stimulatory photoperiods ($n = 0$) would then be quantitative evidence for a "carry-over effect." Conversely a value of k less than that obtained with daily stimulatory photoperiods would be evidence for significant gonadal regression between the days with long daily photoperiods.

From previous experience with *Zonotrichia leucophrys gambelii* we selected a 20-hour photoperiod (followed by four hours of dark) for the long day (L) and an 8-hour photoperiod (followed by sixteen hours of dark) for the short day (S).

Experiment I. Birds were captured from migrant flocks during early and mid-September. On 30 October they were transferred indoors and held on 8-hour daily photoperiods (09:00–17:00) until exposure to the experimental lighting regimens. Six groups of 15–20 birds each were submitted to the following treatments: *Group L_1*, a long daily photoperiod every day beginning on 16 November; *Group L_2*, the same regimen but beginning on 24 December; *Group L S,* alternating days with long and short daily photoperiods; *Group L 2S,* repeated cycles of one day with long daily photoperiod followed by two days with short daily photoperiods; *Group L 3S,* repeated cycles of one day with long daily photoperiod followed by three days with short daily photoperiods; *Group L 5S,* repeated cycles of one long day followed by five days with short daily photoperiods. Control Group L_2 was necessary because of the increase in photosensitivity that occurs as a function of time held on short daily photoperiods (Laws, 1961; Farner, 1962; Farner and Follett, 1966). The number of cycles, varying from 20 for L_1 and L_2 to 10 for Group L 3S, was determined by the rate of testicular growth. All birds were killed when the combined testicular weight was about 100 mg. or when the ovarian weight was about 30 mg. and therefore within the linear portion of the logarithmic growth curve (Farner and Wilson, 1957; Farner *et al.,* 1966). The control birds were killed at 10:00, one hour after the beginning of the photoperiod. All other birds were killed at 10:00 on the first short day in their respective cycles.

Experiment II. In principle, this experiment was similar to Experiment I except that more highly photosensitive birds were used. First-year males were captured during autumn migration or from the over-wintering population in the Snake River Canyon near Pullman. They were moved from outdoor aviaries to indoor cages and 8-hour daily photoperiods on 29–31 January. Subsequently they were divided into four groups (12–14 per group) as follows: Group L, Group L 2S, Group L 3S and Group L 5S. The photoperiodic schedules were identical with those for the corresponding groups in Experiment I. The experimental photoperiodic regimens were begun on 11 March.

Experiment III. This experiment was an attempt to identify the periods of synthesis and release of gonadotropin in birds subjected to a photoperiodic cycle with both long and short daily photoperiods. The particular regimen selected was L 3S; the experiment was begun with 39 first-year birds on 11 March. After ten complete cycles birds were killed on the following schedule: At the beginning and

Table I

The effect of alternation of long days and short days on the rate of testicular growth in Zonotrichia leucophrys gambelii

Group	Experiment I		Experiment II	
Photoperiodic cycle*	Rate of testicular growth (*k*)** days^{-1}	Rate of testicular growth (*k*)*** cycles^{-1}	Rate of testicular growth (*k*)** days^{-1}	Rate of testicular growth (*k*)*** cycles^{-1}
L_1	0.099 ± 0.012	0.099	0.111 ± 0.011	0.111
L_2	0.106 ± 0.010	0.106	——	——
L–S	0.087 ± 0.009	0.173	——	——
L-2S	0.072 ± 0.007	0.216	——	——
L-3S	0.064 ± 0.008	0.256	0.065 ± 0.005	0.260
			0.064 ± 0.006****	0.256****
L-5S	0.031 ± 0.018	0.183	0.034 ± 0.013	0.204

* L = "long day" (20 hours light, 4 hours dark); S = "short day" (8 hours light, 16 hours dark).

**k = (log W_t − log W_o)/t where W_o is gonadal weight at day O, W_t is gonadal weight at day *t*, and *t* is time in days. Mean and 95% confidence limits.

*** k with *t* measured as number of photoperiodic cycles.

**** From Experiment III.

the end of the long daily photoperiod in the 11th cycle, and at the ends of the short photoperiod and the long dark period of the first short day thereafter, and at the beginning and the end of the short photoperiod and at the end of the long dark period on the final short day of the cycle (Fig. 3).

Experiment IV. This experiment was designed to determine the time required after the discontinuation of a stimulatory photoperiod for gonadal regression to become apparent. A group of 38 males, drawn from the same population as in Experiment II, was subjected to 20-hour daily photoperiods and was then changed to 8-hour daily photoperiods. Samples of eight were taken before (09:00) and after (05:00) the last long photoperiod and at the beginning of the short daily photoperiod (09:00) on the 3rd, 7th, and 11th days after discontinuation of long daily photoperiods.

Materials and Methods

The experimental birds were captured with Japanese mist nets and held under natural conditions of photoperiod and temperature in large outdoor aviaries until they were moved into small indoor cages in constant condition rooms. Only first-year birds were used. Drinking water and a nutritionally adequate chick-starter mash were freely available. Illumination indoors was provided by incandescent lamps; intensity at the cage floor was at least 400 lux. The temperature was maintained at 21 ± 1° C.

In all experiments the birds were killed by decapitation. Gonads were removed and preserved in a mixture of 10% formaldehyde, 10% acetic acid, 30% ethanol, and 50% water v/v. After five days they were transferred to 70% ethanol; five days later they were weighed on a precision torsion balance. Pituitaries were removed immediately after killing and placed in dry acetone which was changed

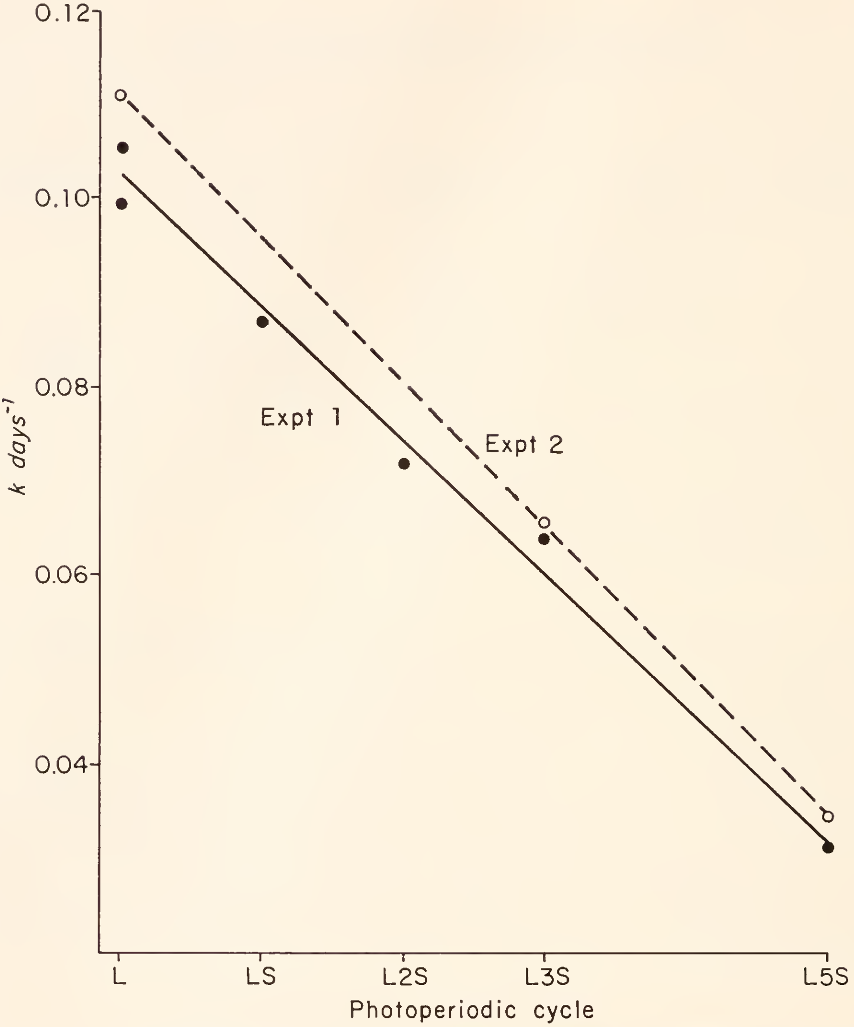

FIGURE 1. Testicular growth rates of *Zonotrichia leucophrys gambelii* as a function of short days (8L 16D) interposed with long days (20L 4D). The birds used in Experiment II had been held longer on short days and were therefore more photosensitive. —— Experiment I. - - - Experiment II.

twice over the next four days. The glands were then air-dried and stored *in vacuo* at 0° C. over phosphorus pentoxide until assay.

Gonadotropins were assayed by the method of Breneman *et al.*, (1962) as modified by Follett and Farner (1966). Glands from several birds were normally pooled and a single assay of the 2 + 2 type performed with 8–10 chicks at each dose level. The results from each assay were subjected to a full analysis of variance (Bliss, 1952). Potency estimates are given, together with 95% confidence

TABLE II

The effect of alternation of long days and short days on the rate of ovarian growth in Zonotrichia leucophrys gambelii Experiment I

Group Photoperiodi cycle*	Rate of ovarian growth (k)** days^{-1}	Rate of ovarian growth (k)*** days^{-1}
L_1	0.034 ± 0.010	0.034
L_2	0.043 ± 0.009	0.043
L-S	0.029 ± 0.005	0.057
L-2S	0.028 ± 0.006	0.084
L-3S	0.022 ± 0.005	0.087
L-5S	0.013 ± 0.003	0.052

* See Table I.

** $k = (\log W_t - \log W_0)/t$ where W_0 is gonadal weight at day O, W_t is gonadal weight at day t and t is time in days. Mean and 95% confidence limits.

*** k with t measured as number of photoperiodic cycles.

limits and the index of precision (λ). The latter is the ratio of the standard deviation to the slope of the dose-response curve. The values of λ compare favorably with other gonadotropin assays. When gonadotropins were estimated throughout the L3S cycle in Experiment III individual glands were assayed (2 + 1 assay). The potency values for each determination were used and subjected to standard statistical procedures for calculations of means and standard errors; comparisons of means were made by Fisher's t test.

RESULTS AND DISCUSSION

The testicular growth rates in Experiments I and II are shown in Table I. The difference in rates between groups L_1 and L_2 in Experiment I and between L_1 of Experiment I and L_2 of Experiment II, although in themselves not statistically significant, are nevertheless consistent with previous observations of increasing photosensitivity as a function of time of exposure to short daily photoperiods (Laws, 1961; Follett and Farner, 1966). As expected, the testicular growth rates, expressed as days $^{-1}$, decreased as a function of the number of short days per cycle (Fig. 1; for Experiment I, $r = 0.98$, $P < 0.01$). However, the slope of the line is small so that the testicular growth-rate constant (in cycles $^{-1}$) is a positive function of the number of short days per cycle up to a maximum for Group L 3S (Table I). The growth-rate constant (in cycles $^{-1}$) for Group L 5S was smaller than that for Group L3S but nevertheless greater than those of the L groups. The ovarian growth-rate constants showed the same pattern although characteristically lower (Table II). The responses of the females were consistent with the hypothesis (Farner *et al.*, 1966) that up to an ovarian weight of about 50 mg. the photoperiodic control mechanism is similar to that of males.

Experiment IV, which was designed to test for testicular regression, showed that the mean weight of the testes increased from 95.6 mg. at the end of the long daily photoperiods to 131.7 mg. after three short days and 146.7 after seven short days. Regression was detected only by eleven short days when the mean testicular

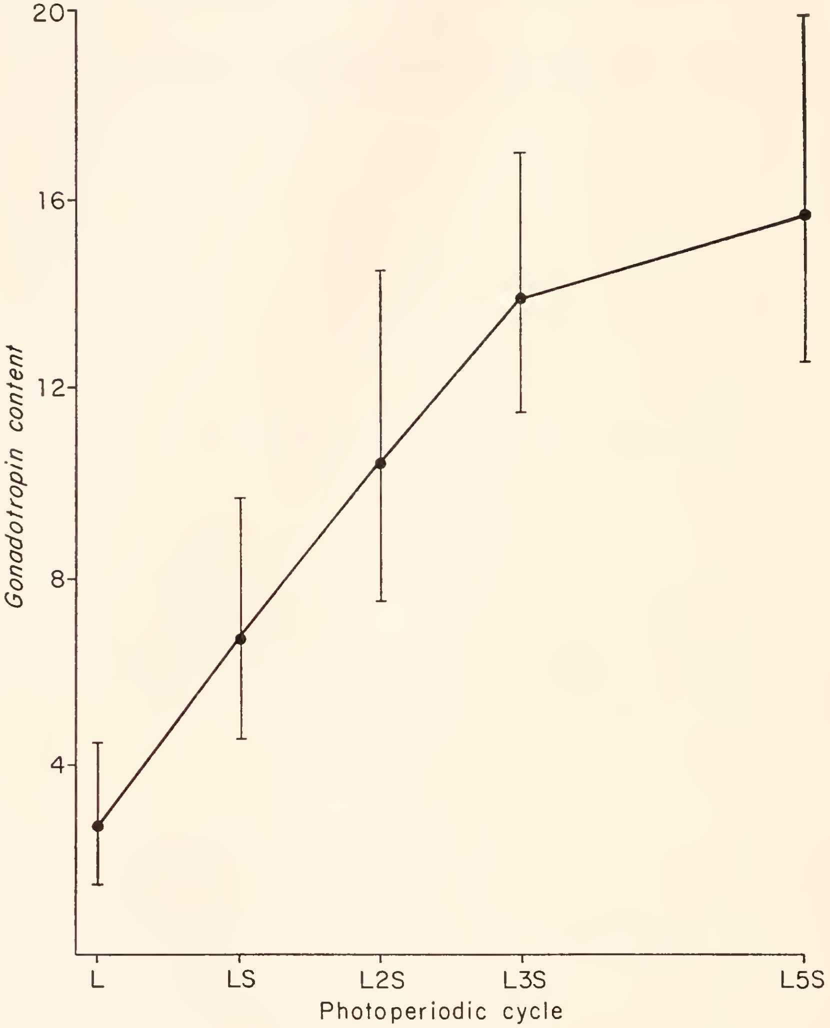

FIGURE 2. Gonadotropin content of the anterior pituitary gland of males (*Zonotrichia leucophrys gambelii*) in μg. equivalents of NIH-LH as a function of short days (8L 16D) interposed with long days (20L 4D). Vertical bars define the 95% confidence limits.

weight was 76.7 mg., a value significantly lower ($P < 0.05$) than the weight after seven short days.

A recent series of ingenious experiments by Wolfson (1966) inject a further parameter into the performance of photoperiodic mechanisms. With *Junco hyemalis* it was found that the photoperiodically induced gonadal growth initiated with 16L 8D continued for as long as 18 days in uninterrupted continuous darkness, during which time the birds showed a long-day circadian periodicity in motor activity. Birds that were changed to a short-day regime (9L 16D) instead of

Table III

The effect of alternation of long days and short days on the concentration of gonadotropin in the anterior pituitary gland of male Zonotrichia leucophrys gambelii

Group	Experiment I		Experiment II	
Photoperiodic cycle*	Gonadotropin Content per gland** μg. equivalents	λ***	Gonadotropin Content per gland** μg. equivalents	λ***
L_1	2.7 (1.6– 5.0)	0.195	16.7 (11.5–21.9)	0.159
L_2	6.2 (4.6– 9.6)	0.152	——	——
L-S	6.7 (4.6– 9.8)	0.107	——	——
L-2S	10.4 (7.1–15.2)	0.108	——	——
L-3S	13.9 (11.5–16.9)	0.117	21.8 (15.4–29.6)	0.174
L-5S	15.7 (12.4–20.1)	0.121	17.4 (10.5–24.5)	0.213

* See Table I.

** Mean with 95% confidence limits, μgram equivalents.

***Index of precision. See text.

total darkness underwent gonadal regression. Unfortunately we are unable to perform comparable experiments with *Zonotrichia leucophrys gambelii* since it seems impossible for these birds to feed and drink adequately in continuous *complete* darkness. However, it should be noted that our experiments with *Z. l. gambelii,* involving the shift from long days to short, have given results (gonadal regression) that are apparently similar to those of Wolfson on *J. hyemalis.* The very interesting problem of the synthesis and output of pituitary gonadotropin in total darkness by a bird with a long-day circadian periodicity appears, at present, unapproachable with *Z. l. gambelii.*

Our experiments show that the effect of a long daily photoperiod carries on into the ensuing short days. Therefore, at least in its simplest form, our working hypothesis that each long day results in a fixed logarithmic increment of growth is clearly untenable. However, the apparently linear relationship between growth rate (in cycles $^{-1}$) and the number of days per cycle (Fig. 1), together with the results of Experiment IV, lead to a relatively simple, revised hypothesis. This hypothesis assumes that the gonads are able to derive more growth from the quantum of gonadotropin released by one long day when the long day is followed by one or more short days than when it is followed by another long day because of the longer period for the "use" of the quantum of gonadotropin. However, we do not know definitely whether the quantum of gonadotropin released by a long day is constant or whether it may change as some function of the number of preceding short days. The gonadotropin assays do not supply an answer although the greater accumulation of pituitary gonadotropin in the birds subjected to various L S cycles (Fig. 2; Tables III and IV) and the positive correlation between the gonadotropin concentration in the anterior pituitary and the rate (in cycles $^{-1}$) of gonadal growth (compare Experiments I and II; Fig. 2, Table III) do suggest that the rate of release of gonadotropin is, to some extent, a function of the level of storage of the hormone. The revised hypothesis is illustrated diagrammatically in Figure 4. Under a treatment of daily long photoperiods (Fig. 4a), it is reason-

TABLE IV

The effect of alternation of long days and short days on the concentration of gonadotropin in the anterior pituitary gland of female Zonotrichia leucophrys gambelii Experiment I

Group Photoperiodic cycle*	Gonadotropin Content per gland** μg. equivalents	λ***
L_1	3.5 (1.6– 7.4)	0.195
L_2	9.9 (7.5–15.9)	0.134
L-S	8.9 (7.6–12.1)	0.121
L-2S	14.6 (11.4–19.2)	0.133
L-3S	14.8 (11.4–18.9)	0.148
L-5S	11.4 (8.7–14.5)	0.132

* See Table I.
** Mean with 95% confidence limits, μgram equivalents.
*** Index of precision. See text.

able to assume that gonadal growth is maximal, for the specific duration of the daily photoperiod, and relatively constant logarithmically for about 30 days (Farner and Wilson, 1957; Farner *et al.,* 1966). This is supported by the linear relationship between the logarithm of gonadal weight and time for 25–30 days after the beginning of photoperiodic stimulation, and also by the fact that gonadal growth is normally limited by the rate of secretion of gonadotropin and not by the inherent growth potential of the gonad (Follett, Farner, King and Morton, unpublished). On the other hand, the logarithmic growth increment in an L 3S cycle probably varies greatly from day to day (Fig. 4b). Since release of gonadotropin is presumably induced only by the long day, the gonadal growth rate would reach a maximum at this time or shortly thereafter. However, the gonadotropin has a persistent effect on gonadal growth mechanisms (as indicated by all bioassay techniques) and thus, whereas the rate of gonadal growth decreases through the three short days, growth does not cease immediately after cessation of gonadotropin release. Figure 4 suggests an explanation of the greater gonadal growth rate per cycle for L 3S as compared to L. The area under the curve represents the amount of gonadal growth (growth rate × time); in cycle L 3S this area is greater than in cycle L. Under natural conditions this would not be expressed because of the daily release of gonadotropin. It can become apparent only when non-stimulatory days are used to extend the duration of action of a single daily "quantum" of gonadotropin. In its present semi-quantitative form the revised hypothesis does not describe the mechanism of the protracted effect of the long photoperiod. However, it is significant that the half-life of gonadotropins in plasma (*e.g.* Catchpole, 1964) is relatively short compared with the duration of their effect on the target organs. It is clear that several alternate hypotheses could be proposed to explain the increase in k (in cycles $^{-1}$). Among these is the possibility of a lag in conversion from a long-day circadian periodicity, of the type that Wolfson (1966) has reported to persist on changing to continuous darkness, to a short-day type periodicity. However, the alternates considered by us, in each case, have proven to be more complex than the hypothesis proposed here.

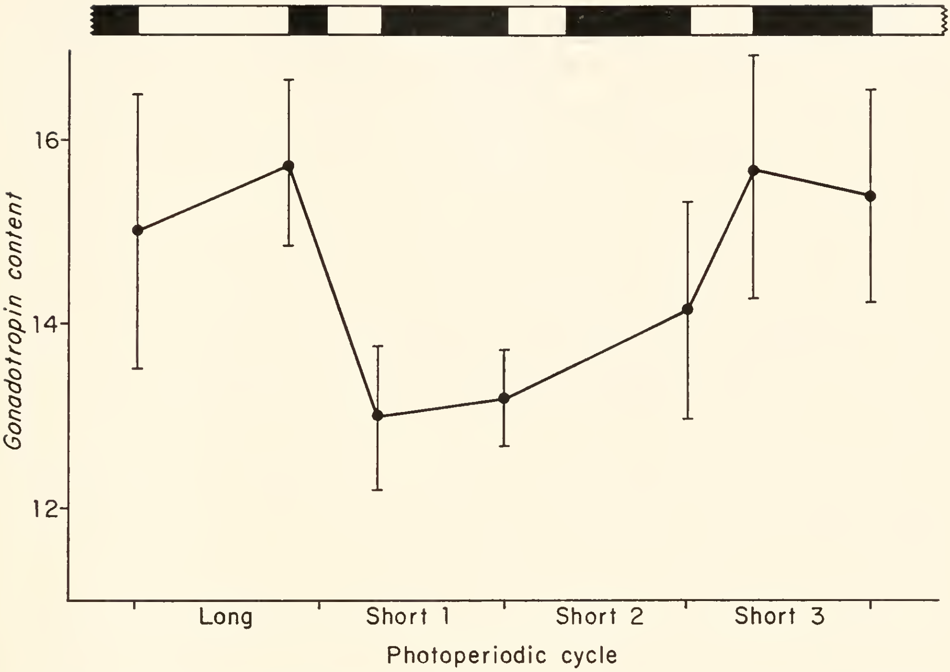

FIGURE 3. Gonadotropin content of the anterior pituitary of males (*Zonotrichia leucophrys gambelii*) in μg. equivalents of NIH-LH in a cycle consisting of one long day (20L 4D) followed by three short days (8L 16D). Black and white bars depict dark and light periods, respectively. Vertical bars define the 95% confidence limits.

In Experiment I there was a close linear relationship between the concentration of pituitary gonadotropin and the number of short days per cycle (Fig. 2). This suggests that synthesis occurs during the short days without release, or at least with a lower rate of release, with a consequent accumulation of gonadotropin proportional to the number of short days. This conclusion is also supported by the ratios of gonadotropin content to the number of days per cycle; these ratios are L, 2.7; L S, 3.3; L 2S, 3.4 and L 5S, 2.6. Particular significance here lies in an apparent separation of the mechanisms that control synthesis and release of adenohypophysial gonadotropin. The absence of a similar relationship in Experiment II is difficult to explain; however, it may also reflect a separation of the mechanisms of control of synthesis and release since we (Follett and Farner, 1966) have shown that the photoperiodic history of an individual bird profoundly affects the storage level of pituitary gonadotropins, a prolonged exposure to short daily photoperiods resulting in a steady increase in concentration (Fig. 2). Thus in Experiment II it is possible that the pituitary gonadotropins were already at maximal level and that further storage was not possible. It is significant in this context that the rates of gonadal growth are essentially similar in Experiments I and II (Table I and Fig. 1), suggesting that the differences in pituitary gonadotropic potency between the groups probably are associated with the control of synthesis rather than with the rate of release.

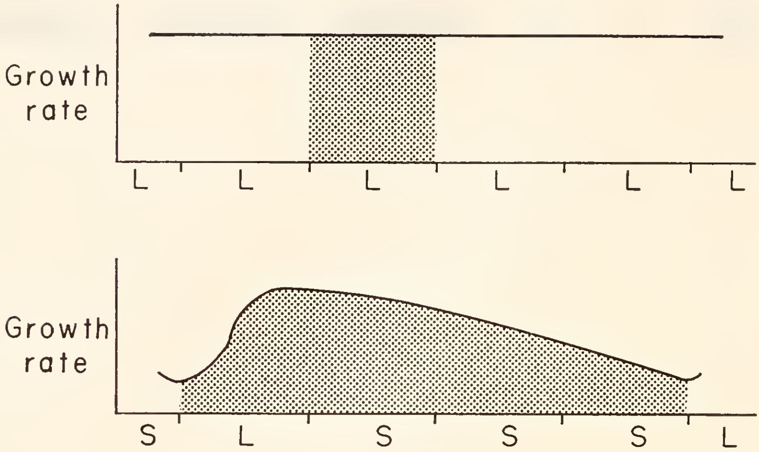

FIGURE 4. Hypothetical gonadal growth rates for *Zonotrichia leucophrys gambelii* under (a) continuous long days (upper) and (b) during a cycle consisting of a long day followed by three short days (lower). The shaded area above is characteristic for each L, *i.e.,* daily. The shaded area below is characteristic of each L 3S cycle.

Experiment III (Fig. 3) was designed to explore more precisely the periods of synthesis and release in an L S cycle and thus assist in the interpretation of Experiments I and II. Although it might be expected that the main release of gonadotropin would occur during the long daily photoperiod no differences were detected in gonadotropins over this time. The simplest explanation must be that rate of synthesis during the long day is equal to the rate of release of hormone with no change in glandular content. During the period of 12 hours following the long daily photoperiod there was a significant drop in pituitary gonadotropins ($P < 0.05$) ; the rate of synthesis at this time can then be assumed to have decreased from the rate during the long photoperiod whereas there must have been continued release. In any case the decrease supports the case of a short-term "carry-over effect" (see Farner and Follett, 1966) of hypothalamic or pituitary origin that causes continual release of hormone into the dark period. Alternatively one could consider that the main release of gonadotropin does not occur during the long daily photoperiod but subsequent to it; this hypothesis seems unlikely since the maximal rate of testicular growth occurs in continuous light (Farner and Wilson, 1957).

During the final two and one-half days of short photoperiods of the cycle there is an increase in adenohypophysial gonadotropin. Again there appears to be at least a partial separation of the controls of the rates of synthesis and release, the former exceeding the latter. These data provide an experimental basis for rationalization of the results of Experiment I in which the concentration of pituitary gonadotropin is directly related to the number of short days per cycle (Fig. 2).

Finally, it seems probable that the increased rate of gonadal growth (in cycles $^{-1}$) in the groups subjected to L S cycles involves a persistent anabolic action of gonadotropin on the gonad, an action that apparently persists for 7–11 days after the long photoperiod. The increase in pituitary gonadotropins as a function of the number of short days per cycle in Experiment I provides a further suggestion of separate control of the synthesis and secretion. Although the exact timing of the release of hormone caused by a stimulatory photoperiod remains to be elucidated, it becomes evident after the end of the long daily photoperiod.

SUMMARY

The characteristics of the gonadal photoperiodic responses in the White-crowned Sparrow, *Zonotrichia leucophrys gambelii,* have been examined by the use of cyclic photoperiodic regimes consisting of a day with a long photoperiod followed by *n* days with short, non-stimulatory daily photoperiod, *n* with the range 0–5; responses measured were the rates of gonadal growth and the concentration of pituitary gonadotropin. The gonadotropic effect of a single 20-hour photoperiod extends through at least seven days with short (8-hour) non-stimulatory photoperiods. The apparently simplest interpretation of the results of the experiments suggests that there must be separate, although perhaps not completely independent, control schemes for synthesis and release of pituitary gonadotropin.

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THE FEEDING AND ECOLOGY OF TWO NORTH PACIFIC ABARENICOLA SPECIES (ARENICOLIDAE, POLYCHAETA)[1,2]

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Lugworms (*Arenicola* and *Abarenicola* spp.) often occur in dense populations on sandy or muddy beaches throughout the world. The two genera are separated on the basis of differences in the prostomium, mechanism of proboscis movement, number of oesophageal caeca, and length of the neuropodia (Wells, 1959). When Wells (1963) summarized the worldwide distribution of lugworms, he noted that, despite certain morphological differences, the mode of life seems to be basically the same for all lugworms. When two species live in the same geographical location, they occur in different habitats. Thus, the sympatric species are ecologically separated.

Most behavioral studies on lugworms have been concerned only with *Arenicola marina* Linné. Wells (1945) described the burrow of *A. marina* as consisting of a vertical tail shaft, a horizontal gallery, and a vertical head shaft. The head shaft is a column of sand loosened by an irrigation current set up by peristaltic waves of the lugworm's body. The sand is consumed at the base of the head shaft and deposited later as fecal castings on the sediment surface near the tail shaft. Wells (1949) recorded the irrigation of the burrow and found it to be cyclical, spontaneous, and predominantly headward. Krüger (1958) observed that *A. marina* could ingest particles suspended in the irrigation current by straining them off in the sediment of the head shaft.

The purpose of my research was to compare the ecology of the two genera of lugworms and of two sympatric species by studying the environment and feeding of two species of *Abarenicola* in False Bay, San Juan Island, Washington. These two species and *Arenicola marina* belong geographically to the same lugworm zone (see Wells, 1963).

The study area (Fig. 1) was centered around the Friday Harbor Laboratories, San Juan Island. Most field work was conducted in False Bay, a roughly circular tide flat (diameter at widest point is 1.2 km.), which consists at low tide of a series of bars and troughs extending from the head to the mouth of the bay. The populations of *Abarenicola pacifica* Healy and Wells and *A. claparedi vagabunda* Healy and Wells are patchy and are separated from each other by a lugworm-

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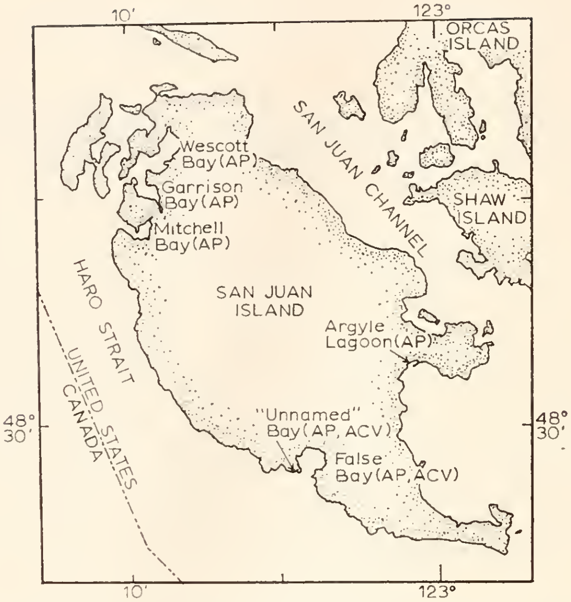
10'
123°
ORCAS ISLAND
SAN JUAN CHANNEL
Wescott Bay(AP)
Garrison Bay(AP)
Mitchell Bay(AP)
SHAW ISLAND
SAN JUAN ISLAND
HARO STRAIT
Argyle Lagoon(AP)
UNITED STATES
CANADA
48° 30'
"Unnamed" Bay(AP, ACV)
False Bay(AP, ACV)
48° 30'
10'
123°

FIGURE 1. Bays sampled for sediment particle size (AP = *A. pacifica* present; ACV = *A. claparedi vagabunda* present).

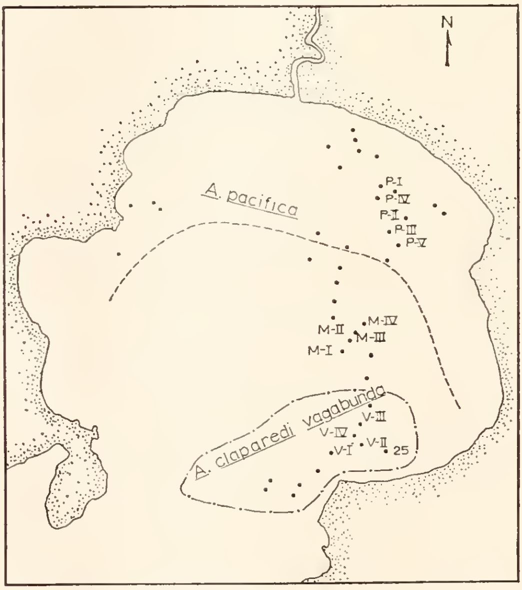
N
A. pacifica
P-I
P-IV
P-II
P-III
P-V
M-II
M-IV
M-III
M-I
A. claparedi vagabunda
V-III
V-IV
V-I
V-II
25

FIGURE 2. Approximate population boundaries in False Bay in summer, 1965. Dots represent sediment sampling locations, and those with Roman numerals represent transplant stations.

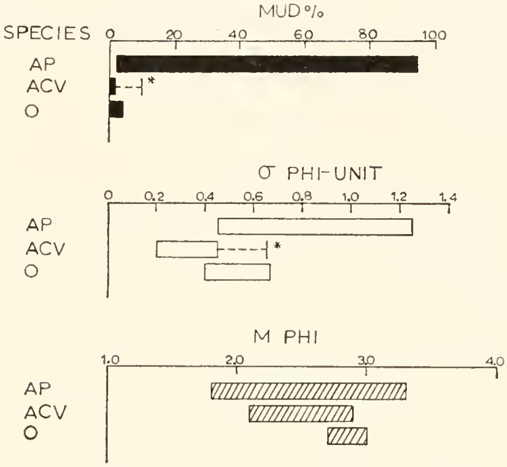

FIGURE 3. Ranges in mud content (Mud %), sorting (σ phi-unit), and mean particle size (M phi) of the sediment inhabited by *A. pacifica* (AP) and *A. claparedi vagabunda* (ACV) and of the sediment from the middle of False Bay (0). (* = a single exception to the ranges.)

free region in the middle of the bay. The population boundaries shown in Figure 2 vary both with season and year, as Healy and Wells (1959) have already noted.

According to Healy and Wells (1959), *A. pacifica* inhabits muddy sand around the margin of False Bay, and *A. claparedi vagabunda* inhabits loose, clean sand at a lower elevation near the mouth of the bay. Sediment rather than tidal exposure appears to govern their distribution. When the two species live in the same location, their relative elevations depend on whether the muddy sediment is higher or lower on the beach than the sandy sediment. Healy and Wells also noted that while the burrows of *A. pacifica* resemble those of *Arenicola marina,* those of *Abarenicola claperedi vagabunda* do not appear to have head shafts or funnel-shaped depressions in the sediment. This may be a result of sediment and water action, as I have observed that in the laboratory the latter species does form head shafts marked by funnel-shaped depressions.

THE ENVIRONMENT OF ABARENICOLA SPECIES

Sediment particle size

Fifty sediment samples were taken from the six locations in Figure 1. The samples were dried and separated into half-phi-unit size fractions and weighed. A phi-unit is the negative logarithm to the base two of the particle diameter in millimeters (Krumbein, 1936). Particle sizes smaller than 0.06 mm. (> 4 phi) were considered mud. Sorting (σ phi-unit) and mean particle size (m phi) were calculated using Inman's (1952) formulae.

Figure 3 shows the ranges from all six bays of the mud content, sorting, and mean particle size for each species and for stations in the middle of False Bay. *Abarenicola pacifica* inhabits a muddier (hence, less permeable) and more poorly sorted sediment than does *A. claparedi vagabunda.* Two small specimens of the latter species were found at station 25 (mud % = 10.0) in False Bay and will be discussed later. The mean particle size is apparently of little importance in separating the habitats of the two species.

Organic content

Total organic matter of surface (0–0.5 cm.) and deep (6–10 cm.) samples from False Bay during spring and summer was measured as loss upon ignition (at 500° C. for 4 hrs.). The range in organic matter in 13 samples from the habitat of *A. pacifica* was 0.5–1.2% (mean = 0.8%; SD = 0.2%). The range in organic matter in 5 samples from the habitat of *A. claparedi vagabunda* was 0.5–0.7% (mean = 0.6%; SD = 0.2%). There was little if any difference between the surface and the deep sediments.

In summary, the environment inhabited by *A. pacifica* is a muddier and more poorly sorted sediment than that of *A. claparedi vagabunda.* Because it lives in a muddier sediment, *A. pacifica* tends to be found in sediment of higher organic content than does *A. claparedi vagabunda,* but it can also inhabit sediment of relatively low organic content.

THE FEEDING BEHAVIOR OF ABARENICOLA SPECIES

Suspension feeding

I attempted to recover, in the castings, carmine particles that had been suspended in the overlying water (using the method of Krüger, 1958) to provide evidence of suspension feeding in *Abarenicola* species. When a red casting appeared, the inside was examined for carmine particles. Although both species could ingest particles suspended in the sea water, there were experimental runs in which no carmine particles appeared in the castings, even after 24 hours.

Sediment feeding

The feeding of individual worms was studied in 0.22-liter glass jars. The manner of feeding on the surface sediment was basically the same as that of *Arenicola marina.* Headward irrigation waves created an upward current of water and of the smaller sediment particles in the head shaft. As the worm crawled backward to defecate or ceased headward irrigation, the head shaft collapsed somewhat. The surface sediment, marked by orange or yellow chalk powder, moved down. The worm then crawled forward and appeared to consume sediment at the base of the head shaft and the cycle began again. Chalk powder appeared in the castings of both species 2 or more hours after its deposition on the surface. The head shaft of *Abarenicola pacifica* and *A. claparedi vagabunda,* like that of *Arenicola marina* (Wells, 1945), represents a subsiding column of surface sand consumed at the base by the worm and renewed at the surface by sedimentation.

The irrigation cycle

Wells (1953) showed that *A. marina* may integrate feeding and defecation into its irrigation cycle. I used Wells' (1953) method to study this behavior in

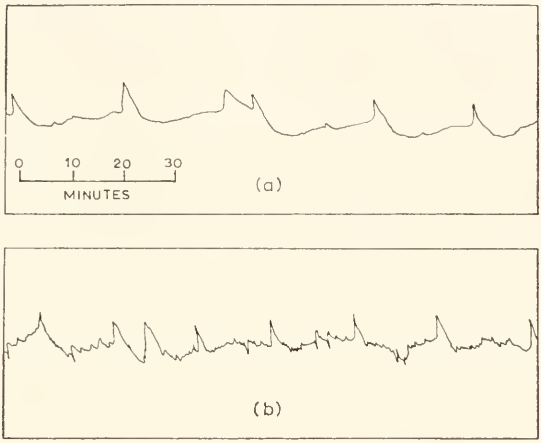

FIGURE 4. Typical irrigation patterns of (a) *A. pacifica* and (b) *A. claparedi vagabunda.*

Abarenicola species. The activities of a worm in a sediment-filled U-shaped tube (the "tail end" opens below the level of a constant level water bath) change the water level in the "head end" of the tube (which opens above the level of the water bath). A float in the "head end" of the tube is attached to a lever bearing a pen (a rise in water level is thus recorded as a downward stroke and usually indicates headward pumping). A narrow-bore capillary at this end just below the level of the water bath was necessary to prevent overflow due to the predominantly headward pumping of both species. Twenty-four individuals of *A. pacifica* and 13 of *A. claparedi vagabunda* (weighing 1–3 g.) were studied.

Figure 4 shows a typical irrigation pattern for each species, to be read from left to right. As the worm backs up the tail shaft, the pen moves upward. At the peak of the curve, the tip of the tail may appear at the surface, but defecation does not always occur. As the worm returns to its original position, the pen falls. The worm then resumes headward irrigation and, apparently, feeding. The average length of the cycles ranges from 10 to 30 minutes and varies considerably among individuals. The only consistent difference between the irrigation cycles of the two *Abarenicola* species is that *A. claparedi vagabunda* traced a more jagged cycle than *A. pacifica.* This difference persisted even when the two species were placed in each other's sediment.

Basically *Arenicola marina, Abarenicola pacifica,* and *A. claparedi vagabunda* have the same mode of feeding. All can feed on particles suspended in sea water, and all feed predominantly on surface rather than deep sediment. They irrigate their burrows in the same cyclical manner, integrating feeding and defecation into this cycle.

THE UTILIZATION OF ORGANIC CARBON

Although the three species have basically the same feeding behavior, there still may be quantitative differences in feeding. The percentage of the organic carbon

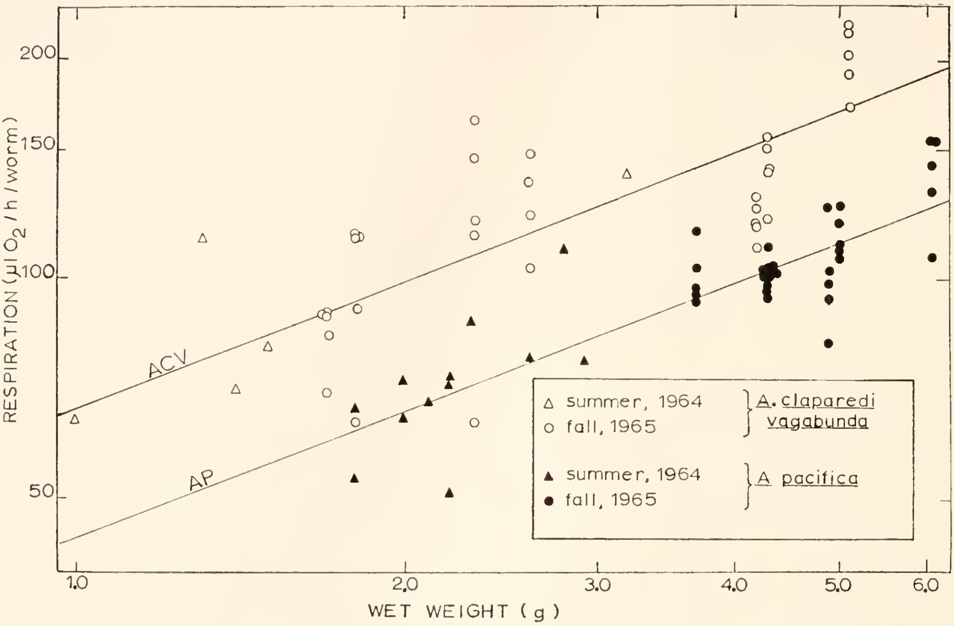

FIGURE 5. Respiration *versus* wet weight for *A. pacifica* (AP) and *A. claparedi vagabunda* (ACV). Values are on a logarithmic scale.

ingested that is needed for maintenance can be calculated for *Abarenicola pacifica* and *A. claparedi vagabunda* from their respiration rate, sediment turnover rate, and the average organic matter content of False Bay sediment.

Respiration rate

The respiration rates of both species were measured in summer, 1964, and fall, 1965, to determine the amount of carbon needed for maintenance. A modification of Conover's (1956) micro-Winkler technique was used. During experiments, each lugworm was placed in an opaque 275-ml. bottle filled with sea water that had been filtered through a glass-fiber filter and equilibrated with the atmosphere. Experimental and control bottles were kept at 12 to 14° C. for 3 hours. The oxygen content of a 25-ml. subsample was determined. Between experiments, the worms were kept in sand from their own habitat.

The results from unstarved worms that were acclimated to laboratory conditions for more than 12 hours are presented in Figure 5 (an open triangle representing 0.8, 73.5 could not be included on this figure). The logarithms of the values of respiration and wet weight were analyzed by covariance analysis. The two species have significantly different respiration rates ($P = \ll 0.01$, with 2 and 78 df). The slopes are not significantly different ($P = 0.10$–0.25, with 1 and 78 df). The slope of the regression lines (b = 0.58, with 95% confidence interval of 0.48–0.68) is determined from a narrow size range of experimental animals (0.8–6.1 g.) and therefore may not be accurate for the entire size range of the species.

The first few days of starvation do not affect the respiration rate. During summer, 1964, a respiration experiment was run on six *A. pacifica* after 3 days of starvation. There was no noticeable decrease in the respiration rate. Six individuals of *A. claparedi vagabunda* were starved for 3, 8, and 10 days. Only after 10 days of starvation was there a noticeable (about 30%) decrease in the respiration rate.

Sediment turnover rate

The quantity of sediment passing through the gut per unit time was measured to assess the feeding rate of both species in False Bay during summer. Eight individuals of each species were used. Each worm was placed in a 0.22-liter jar (10 cm. high) in sediment from its own habitat. The castings were collected three or four times each day, dried, and weighed. In the first set of experiments, the jars were kept continuously under water (10–12.5° C.) to simulate continuous high tide. The same worms were used in the second set of experiments, but under simulated tidal conditions of False Bay. Both sets of experiments lasted for 9–11 days. To allow the worms to adjust to conditions, only the results from the last 5 days were used. Five hours of "low tide" was alternated with 7 hours of "high tide" for the experiments with *A. pacifica* to approximate the average exposure of the population. Low tide was simulated by draining the water bath and siphoning water from the jar and half the water from a vertical column in the sediment. The average exposure of the *A. claparedi vagabunda* population was estimated to be once a day for 2.5 hours and the jars were drained accordingly. Water was siphoned only from the sediment surface, simulating the loose watery sand near the mouth of False Bay. The rise in sediment temperature during "low tide" (13° C. average) roughly simulated that occurring in False Bay at about 10 cm. depth. At the end of the experiments, the sediments in six jars were sampled for organic matter.

Under continuous high-tide conditions, the average sediment turnover rate of *A. pacifica* was 3.8 g./day and that of *A. claparedi vagabunda* was 12.4 g./day. Under tidal conditions, the respective rates were 3.4 g./day and 9.4 g./day. Within the size range of worms used (1.0–3.5 g.) there was no relation of sediment turnover rate to size. Although I noticed that the castings left during "low tide" were smaller than at "high tide," there was no significant difference in turnover rate between conditions of "tides" and "no tides" ($P = 0.10$–0.25, with 1 and 28 df). However, there was a significant difference between the turnover rates of the two species ($P = \ll 0.01$, with 1 and 28 df). There was no significant interaction between tidal conditions and species ($P = > 0.25$, with 1 and 28 df).

Because experimental conditions were meant to resemble the environmental conditions of the two species' habitats in False Bay, the organic content of the sediment could not be kept equal for both species during the experiment. As the organic content was lower in the *A. claparedi vagabunda* sediment (0.4%) than in the *A. pacifica* sediment (0.8%), one might expect a higher sediment turnover rate for the former species. However, the observed higher activity and the higher respiratory rate of *A. claparedi vagabunda* suggest that there is a real difference in sediment turnover rate between species.

TABLE I

Utilization of organic carbon by a 2-gram lugworm

Species	Respiration rate at 12°C. (μl. O_2/hr.)	Carbon needed* (mg. C/day)	Average carbon content of sediment** (mg. C/g.)	Average sediment turnover rate (g./day)	Carbon ingested (mg. C/day)	Carbon needed / Carbon ingested (%)
A. pacifica	66	0.65	4.0	3.6	14	4.6
A. claparedi vagabunda	99	0.98	3.0	11	33	3.0

* An RQ of 0.8 is assumed.
** Organic carbon is assumed to be half the organic matter.

Utilization of organic carbon

The percentage of carbon utilized by the two species is calculated in Table I. *A. pacifica* needs 4.6% and *A. claparedi vagabunda* needs 3.0% of the carbon ingested (from sediment feeding only). It appears that the lugworms remove little of the organic carbon available from the sediment, and thus their food requirements are fairly low. One must consider, however, that not all of the carbon compounds passing through the gut could be broken down by the enzymes of the lugworm. George (1964) found that under optimal conditions the enzymes of the polychaete *Cirriformia tentaculata* (Montagu) digested only 14% of the ingestible organic matter. Even if the enzymes of the lugworm could digest only 10% of the organic carbon ingested, only 30–50% of this would be needed for maintenance. Because the time required for a dense population of 50 *A. pacifica*/m.^{2} to turn over all sediment to 10 cm. depth is calculated to be about 800 days, it is unlikely that the lugworms would ever deplete the food supply in the sediment. In addition, suspension feeding would increase the amount of carbon ingested. It is probable, therefore, that *A. pacifica* inhabits a muddier sediment than *A. claparedi vagabunda* because of factors other than the high organic content of mud.

TRANSPLANTATION EXPERIMENTS

Both species were transplanted into their own habitat, each other's habitat, and the lugworm-free middle portion of False Bay in order to determine whether the difference in habitats influences the adult worms. The stations at these three locations are marked in Roman numerals in Figure 2.

Ten worms of one species were placed on the sediment surface within the rim of cylinders of 1-mm. mesh plastic screen (height, 30 cm.; diameter, 30 cm.), which had been submerged to 27 cm. depth in the sediment. These cylinders allowed for natural drainage while preventing the escape of the lugworms. In each of the three locations, two cylinders were used for *A. pacifica* and two for *A. claparedi vagabunda*. Two series of these transplants were conducted on bars (above water level at low tide) and three series in troughs (below water at all times). Transplantation of *A. claparedi vagabunda* was discontinued after the first two series because even the control transplants did not survive well. At each sufficiently low

TABLE II

False Bay transplants

Habitat*	Sediment settled or unsettled**	Survival values*** (after 14 days)		Mean
		Bars	Troughs	
		A. pacifica		
AP	S	9; 5;	2; 6	5.5
	U	9; 8;	7; 5; 2; 3	5.7
M	S	0; 0;	0; 0; 0; 0	0
	U	0; 0;	0; 0	0
ACV	S	0; 0;	0; 0; 0	0
	U	4; 1;	2; 3; 3	2.6
		A. claparedi vagabunda		
AP	S	1; 0		0.5
	U	3; 0		1.5
M	S	5; 0; 1; 1		1.6
ACV	S	0; 0		0
	U	6; 3		4.5

* AP = in the habitat of *A. pacifica*; M = in the middle of False Bay; ACV = in the habitat of *A. claparedi vagabunda*.

** S = sediment has been allowed to settle for a day or more. U = sediment has not been allowed to settle.

*** Survival values represent the highest number of castings counted during the last spring tide period. For most "0" values, castings were never noticed after transplantation.

tide, the numbers of castings within each rim were counted, and the survival value for each cylinder was taken as the highest number counted during the week of spring tides. After about one to two weeks, the survival values leveled off, and statistical analyses were performed on the results obtained at the end of 14 days (except for the first series of transplants, which was not revisited until about a month later). In about half the experiments with *A. pacifica,* the worms were transplanted several days after the cylinders had been submerged, and thus planted in "settled" sediment rather than in freshly dug sediment. This was to determine the possible effect of physical factors.

The data of the transplantation experiments are summarized in Table II. The results obtained on bars and in troughs were not significantly different and are therefore combined. A *t*-test showed a significant ($P = < 0.01$) difference in survival of *A. pacifica* between settled and unsettled sand in the habitat of *A. claparedi vagabunda.* Settling apparently has no effect on *A. pacifica* in its own habitat or in the middle of False Bay. An analysis of variance showed that there is a significant ($P = < 0.01$) difference in survival among habitats in unsettled sediment.

The *A. claparedi vagabunda* transplants did not survive well even in their own habitat. There is no significant difference in survival among any of the habitats. However, even in settled sediment some worms were able to survive in the habitat of *A. pacifica* and some in the middle of False Bay. Therefore, it appears that the adults of *A. claparedi vagabunda* are able to survive in other habitats. The difference between survival of this species in settled and unsettled sand in its own habitat was not significant ($P = 0.05–0.10$), but these are the results of only two series of transplants.

In the laboratory, ten worms of a species were placed in a sediment-filled aquarium (20 by 30 by 15 cm.) under water for 1 to 6 weeks. Even after 6 weeks in sediment from the middle of False Bay, most individuals of *A. pacifica* were still alive. Both species could survive in the laboratory in sediment from any of the three locations in False Bay.

Some conclusions may be made from the contradictory results of the laboratory transplants. In the laboratory, conditions were the same as in the troughs of False Bay except for the absence of water action and of possible predation by birds and fish. Water action is the more likely explanation for the low survival in some field transplants. *Abarenicola pacifica* has been found only in sediment with more than 1.7% mud, and therefore is subject to less action by waves or currents than is *A. claparedi vagabunda,* which is usually found in a cleaner sediment. The inability of *A. pacifica* to survive in the habitat of *A. claparedi vagabunda* might then be due to the increased water action in this environment. The higher survival of *A. pacifica* in the habitat of *A. claparedi vagabunda,* when placed in unsettled sediment, may be related to the establishment of the burrow. The sediment becomes settled after a period of high tide, but before this time the *A. pacifica* have probably established their burrow. The mud content at two of the transplant stations (1.7%, 2.3%) in the middle of False Bay is within the range in which *A. pacifica* is found, so water action alone cannot account for the inability of *A. pacifica* to survive in this habitat.

The observed distribution of *A. claparedi vagabunda* in sandy sediment is probably a result of larval ecology. The finding of two specimens (station 25) in 10.0% mud and the survival of some worms both in the middle of False Bay and in the habitat of *A. pacifica* suggest that adults of *A. claparedi vagabunda* are capable of living in these locations.

Discussion and Conclusions

The feeding behavior of two North Pacific *Abarenicola* species is basically the same as that of *Arenicola marina.* The representatives of both genera feed predominantly on surface rather than deep sediment, are capable of feeding on particles suspended in the sea water, and integrate feeding, defecation, and irrigation into their activity cycle.

There is a definite quantitative difference in feeding between the two *Abarenicola* species. The higher activity of the irrigation cycles, the higher respiratory rate, and the higher sediment turnover rate demonstrate that *A. claparedi vagabunda* is generally more active than *A. pacifica.* The utilization of organic carbon is nearly the same for the two species and they need little (3–5%) of the organic carbon ingested. Although the lugworms cannot digest all of the organic carbon ingested, calculations show that even in dense lugworm populations the food supply in the sediment probably would not be depleted. Of what value, then, is the suspension feeding? Krüger (1964) believes that suspension feeding may contribute a high percentage of the food requirements of *Arenicola marina*. Because the carbon requirements of the species studied here are low, either sediment or suspension feeding probably could supply much of the needed organic carbon. We must first determine what percentage of the organic carbon in the sediment can be assimilated by lugworms before assessing the value of suspension feeding. If the organic content of the sediment becomes very low or is chiefly in a form unavailable to the enzymes of the lugworms, particles suspended in the sea water would be an important supplement to the lugworms' diet.

If these two species of *Abarenicola* are not limited by food, their distribution should be a result of larval ecology, physical factors, and/or predation and parasites. The transplant experiments suggest that physical factors, probably the action of waves and currents on the sediment, render the sediment of sandier habitats unsuitable for *A. pacifica*. Thus, this species is found in a muddy habitat, where water movement is comparatively lower. On the other hand, the transplant experiments suggest that larval settlement and survival may be responsible for the usual occurrence of *A. claparedi vagabunda* in clean sand. Because, for the reasons proposed above, *A. pacifica* and *A. claparedi vagabunda* live in sediments of differing mud content, they should never compete with each other—even when sympatric as in False Bay.

Why should three species of two genera have the same type of feeding behavior? The amount of food available from sediment and suspension feeding is large compared to the amount needed. At low tide aerial respiration and defecation may be fitted to the activity cycle, and at high tide suspension and sediment feeding, defecation, and burrow irrigation are integrated. This appears to be a feeding mechanism that should be successful throughout the range of lugworm habitats. There should be no selection pressure to change a successful way of feeding. Competition for food should not have caused a divergence in feeding mechanisms because, even if the two species had occurred at the same point in the past, food probably was not limiting. It is probable, therefore, that this feeding behavior has been retained throughout lugworm evolution.

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SUMMARY

1. A comparison of the habitats of two sympatric lugworms shows that *Abarenicola pacifica* lives in a muddier and more poorly sorted sediment than does *A. claparedi vagabunda*.

2. Both species, like *Arenicola marina*, are capable of suspension feeding, feed

predominantly on surface rather than deep sediment, and integrate feeding and defecation into their irrigation cycle.

3. Respiration and sediment turnover rates indicate that *Abarenicola claparedi vagabunda* is more active than *A. pacifica*. Both species seem to need little (3–5%) of the organic carbon ingested.

4. Transplantation experiments suggest that physical factors rather than availability of food may limit *A. pacifica* to muddier sediment and that the distribution of *A. claparedi vagabunda* may be a result of larval settlement and survival.

5. It is proposed that this mode of feeding has been retained throughout lugworm evolution because the observed feeding behavior should have been successful throughout the range of lugworm habitats, and because of the probable lack of interspecific competition for food.

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PURIFICATION OF THE BRAIN HORMONE OF THE SILKWORM BOMBYX MORI

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The endocrine function of the insect brain was first suggested by Kopeć as early as in 1922 in the gypsy moth, *Lymantria dispar.* Years later, Wigglesworth (1940) clearly demonstrated that the molting of the bug, *Rhodnius prolixus,* was initiated by a hormonal factor originating from the dorsal region of the protocerebrum containing the neurosecretory cells. Since then, numerous studies have clearly defined the function of the insect brain hormone (BH). Thus BH stimulates the prothoracic glands to secrete the prothoracic gland hormone, ecdysone (*e.g.,* Williams, 1947, 1952; Wigglesworth, 1952) which then is thought to act directly on the cells of the various tissues to provoke the growth and metamorphosis of the insect as a whole. BH thus occupies a central position in the endocrine network which controls the post-embryonic development of insects.

The chemical study of BH has been reported from three laboratories with contradictory results. An extract possessing the BH activity was first prepared by Kobayashi and Kirimura (1958) from brains of the silkworm, *Bombyx mori.* Later Kobayashi and his associates (Kobayashi *et al.*, 1962a, 1962b; Kirimura *et al.*, 1962; Saito *et al.*, 1963) obtained the active substance in a crystalline form and identified it as cholesterol. Gersch and his associates (Gersch *et al.*, 1960) obtained the crystalline neurohormones from the entire central nervous tissue of the cockroach, *Periplaneta americana;* the BH activity was detected in one of them, neurohormone D_1 (Gersch, 1961, 1962). In 1960 we obtained a potent watery extract from *Bombyx* brains (Ichikawa and Ishizaki, 1960) and, later, concluded that this active principle was a protein (Ichikawa and Ishizaki, 1963). The present paper deals with the results of the further purification of BH. About 8000-fold purification, on the basis of protein measurement, was achieved and the hormone was proved to consist of chromatographically highly heterogeneous molecules, the molecular weight of the major components varying from 9000 to 31,000.

MATERIALS AND METHODS

Preparation of assay pupae

Debrained pupae of the Eri-silkworm, *Samia cynthia ricini,* were used for the bioassay of BH. This species is non-diapausing but the arrest of development is brought about when the brain is surgically removed from pupae within 1 day after the pupal molt.

BH was distinguished from the prothoracic gland hormone (ecdysone) by tests on isolated abdomens of *S. cynthia ricini.* The latter were prepared by cutting pupae into halves between the 3rd and 4th abdominal segments and sealing the posterior half with melted paraffin.

Debrained diapausing pupae of the swallowtail, *Papilio xuthus,* were also used to assay BH. The diapausing pupae were prepared by rearing larvae in a 7-hour photoperiod and the brain extirpation was carried out within several days after pupation. The pupal diapause in this species has been proved to be due to the failure of the brain to secrete BH, as in other lepidopteran species (Ichikawa and Ishizaki, 1958).

Source of BH extract

Bombyx brains were used as extraction material. In the previous communication 5 devitalized *Bombyx* brains were found sufficient to provoke adult development when implanted into a *Samia* assay pupa (Ichikawa and Ishizaki, 1960). To see if a difference exists in the BH activity of the brain at successive developmental stages, 5 completely desiccated brains of *Bombyx* (at the ages of 0, 2, and 4 days after the 4th larval molting, mature larva, prepupa, 0, 3, and 8 days after the pupal molting, and newly emerged adult) were assayed by implanting them into each of the assay pupae. All were active, indicating that no stage-difference existed in the BH activity present in the brain, at least within the range of sensitivity of our assay. Therefore pupae of mixed ages, ranging from shortly after pupation to just prior to adult emergence, were utilized.

Although it was a tedious job to collect the brains, an advantage was the "mechanical" purification, since BH was contained only in the brain in an appreciable titer, as far as examined so far. The "brain" in the present experiments denotes the brain plus a certain amount of neighboring tissues which were not carefully removed in order to reduce the investment of time and labor.

The brains were stored frozen until extracted. They may be stored frozen for at least 1 year with no appreciable loss in BH activity.

Injection

The materials to be tested were injected into assay pupae at the dorso-lateral site of the 4th abdominal segment after anaesthetizing them with ethyl ether. Each pupa received routinely 0.02 ml. of the test solutions. The puncture was sealed with melted paraffin and the injected pupae placed at 25° C.

Penicillin (Kaken Co. Ltd., 200,000 international units/ml. deionized water) and the saturated phenylthiourea (an inhibitor of melanization) were added to the test materials, in a proportion of 5 μl. of each to 0.4 ml. of the test materials. Adding phenylthiourea was essential in the early steps of purification, for the injection otherwise caused occasionally blackening of the blood followed by the death of the assay pupae. Surviving pupae, in this case, often did not develop after injection of BH. This tendency to yield false negative results was markedly enhanced when the extracts were subjected to the first ammonium sulfate precipitation. The reason for this is not clear, but it might be that the extract contains a tyrosinase inhibitor which is removed by the ammonium sulfate fractionation.

TABLE I

Fate of 120 debrained Samia pupae kept at 25° C. after brain removal

Days after operation	0	24	25	27	28	29	30	31	36	57	63	67	70	85	90	100–200	200–300	300–400	400–500
No. emerged as adult moths	—	1	1	—	2	2	2	1	1	1	—	—	1	—	—	2	9	17	15
No. died without apparent adult development	—	—	1	3	—	1	—	—	—	—	1	1	—	1	1	5	14	16	20
No. surviving as pupae	120	119	117	114	112	109	107	106	105	104	103	102	101	100	99	92	69	36	1

Nature of assay pupae, judgment of positive response, and definition of "Samia unit" of BH

The fate of the 120 debrained *Samia pupae* which were monitored for 500 days after brain removal is shown in Table I. Most of them survived for a long time without undergoing adult development, though from time to time some developed spontaneously into brainless adult moths for unknown reasons.

Days required for adult emergence after injection of the materials containing BH varied to some extent, ranging from 17 to 30 days with a mode at 20 days. This period is considerably longer than that between pupation and adult emergence in the normal development, 12–14 days, and was not shortened by high doses of BH. The reason for this delay is unknown but it might be either that a continuous supply of the hormone is necessary for pursuing the normal time course of development or that the metabolic level, once lowered in the debrained resting pupae, needs a supplemental time before recovering the normal level ready for the adult development to start. The moths emerged after injection were of completely adult morphology as far as externally examined, the only anomaly being the failure of the wings to spread out fully.

In the present study, 5 pupae were routinely used for the assay of each experimental lot. The assay result was regarded as positive when more than 3 pupae emerged as adult moths. BH activity was quantified by assaying a series of 2-fold-diluted solutions of the materials. Dilution was made with either deionized water or appropriate buffers. One *Samia* unit of BH was defined as the minimum amount necessary to cause adult development in one assay pupa. An example of the results, to show the mode of response of the pupae to serially diluted BH, is given in Table II. Generally, a boundary concentration (8-fold dilution in this case) where only certain pupae responded could be separated from the clearly positive and negative groups. Thus, in this case, one can know that one *Samia* unit was present in 0.02 ml. of the 8-fold-diluted solution. It is seen that one pupa did not respond to the original solution. Such exceptional individuals were occasionally encountered.

Chemical procedures

All of the chemicals used were of the highest purity commercially available. Aqueous solutions were prepared with deionized water. Sephadex gel (Pharmacia), DEAE-cellulose (Serva, 0.65 mEq/g.), and CM-cellulose (Serva, 0.80 mEq/g.) were washed and packed in columns with standard procedures. Cytochrome *c* and crystalline α-chymotrypsin were the gifts of Dr. T. Kato of the

TABLE II

An example of the assay result to show the mode of response of Samia assay pupae to serially diluted BH

Dilution	No. positive/No. negative	Days required for adult emergence after injection
× 1	4 / 1	19, 19, 20, 20, —
× 2	5 / 0	19, 20, 20, 20, 21
× 4	5 / 0	19, 19, 19, 20, 22
× 8	3 / 2	21, 23, 23, —, —
× 16	0 / 5	—, —, —, —, —
× 32	0 / 5	—, —, —, —, —
× 64	0 / 5	—, —, —, —, —

Department of Botany, Kyoto University, and Eizai Pharmacological Co. Ltd., respectively.

The chemical procedures were carried out either in a cold room (5° C.) or in an ice-water bath. The samples were kept frozen at −18° C. between procedures.

The amount of subtances contained in preparations was expressed in terms of protein amount; protein determinations were made by the use of the Folin phenol reagent with bovine serum albumin as a standard (Lowry *et al.*, 1951). Chromatographic curves were conventionally drawn by the use of optical density at 280 $m\mu$, though even purified preparations did not show a sharp peak at this wavelength.

RESULTS

1. *Purification*

Preparation of crude extract. Preliminary studies on the extractability of BH indicated that it was readily extracted with aqueous solutions, but not with ethyl ether or acetone.

BH was readily extracted with methanol. But after exhaustive extraction overnight with constant stirring with 3 changes of methanol, the residue still possessed an appreciable amount of BH which was readily extracted with saline. This differential extractability will be treated in detail elsewhere. Since all BH was readily extracted with 2% NaCl, this extractant was used in the present study throughout.

One hundred and eighteen thousand brains (fresh weight, 454 g.) were homogenized with 13 times volume (v/w) of acetone and the pellet after centrifugation was dried under reduced pressure. The acetone-dried powder thus prepared (59 *g.*) was extracted 3 times with a total of 2.5 l. of 2% NaCl. Homogenization was performed with a glass homogenizer and centrifugation at 20,000 r.p.m. for 20 minutes. A dark brown extract was obtained. This extract is designated as *Crude Extract.* It contained 992,000 units BH, indicating that one brain contained about 8 units BH.

This extract stimulated adult development also in debrained diapausing pupae of *Papilio xuthus.* The minimum amount necessary to cause development in a *Papilio* pupa was the same as in *Samia,* namely 1 *Samia* unit. Since the average

Table III

Purification of BH

Purification step	BH (*Samia* unit)	Protein (mg.)	Specific activity (BH unit/mg. protein)	Purification: —fold	Yield of BH (%)
Crude extract	992,000	16,100	61.5	1.0	100
Heated fraction	980,000	4,900	200	3.2	98.8
Ammonium-sulfate 30–70% fraction	1,120,000	1,400	800	13.6	112.9
First Sephadex G-100 fraction	1,216,000	800	1,520	24.7	122.6
DEAE-cellulose 0.5 *M* fraction	1,280,000	45	28,400	461	129.0
DEAE-cellulose 6–16 fraction	200,000	4.2	47,600	773	20.2
Second Sephadex G-100 fraction-I	34,650	3.25	10,700	174	3.5
Second Sephadex G-100 fraction-II	165,600	0.32	517,500	8,415	16.7
Second Sephadex G-100 fraction-III	149,850	0.36	416,300	6,769	15.1

body weights of *Samia* and *Papilio* pupae were 2 g. and 0.6 g., respectively, *Papilio* was about 3.3 times less sensitive than *Samia*.

This extract failed to cause adult development in the isolated abdomens of *Samia* after injection of as much as up to 50 *Samia* units, excluding the possibility that the active principle was the prothoracic gland hormone.

Heating. The preliminary experiments revealed that the BH activity was resistant to heating at pH around 6. Since the pH of the *Crude Extract* was already 6.5, it was divided into 4 aliquots of equal volume, each of which (620 ml.) was placed in a 2-liter flask and heated in a 90° C. water-bath with vigorous shaking. Protein coagulation began to appear within 5 minutes and the heating was continued for 3 more minutes. Then the extract was rapidly cooled in an ice-water bath and centrifuged. A strongly yellow solution, pH 6.5, was obtained. All of the BH activity was recovered in the supernatant which is designated as *Heated Fraction* (see Table III).

Fractional precipitation with ammonium sulfate. The preliminary experiments of fractional precipitation with ammonium sulfate revealed that the 50–65% saturation fraction contained most of the BH and the 30–50% fraction also contained it in an appreciable amount (Fig. 1).

Based upon the above results, the *Heated Fraction* was subjected to ammonium sulfate fractional precipitation to collect a 30–70% saturation fraction. To the *Heated Fraction* (2.45 l.) 492 g. of solid ammonium sulfate were very slowly added to reach 30% saturation (saturation in this solution at 5° C. was reached by 0.67 g. ammonium sulfate/ml.). Then the solution was allowed to stand for 30 minutes and centrifuged, the precipitate being discarded. Six hundred and fifty-seven g. ammonium sulfate were then added to reach 70% saturation. After centrifugation, the precipitate was taken up with 112 ml. of deionized water. The precipitate readily dissolved to yield a clear, brown solution. This solution was dialyzed for 24 hours

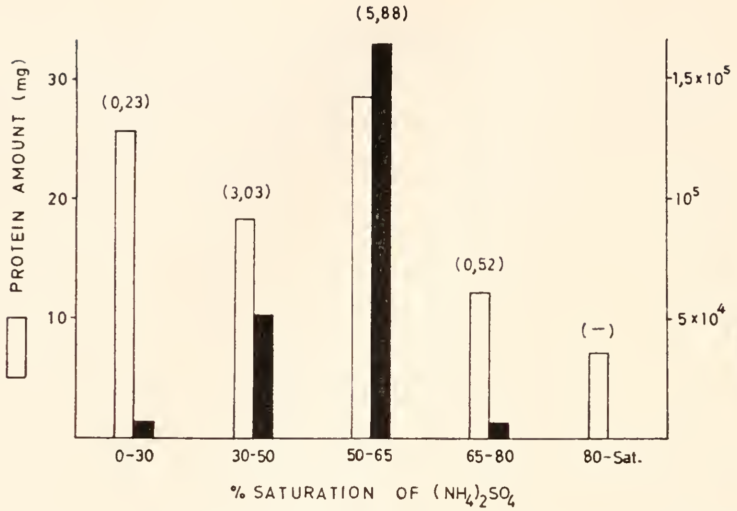

FIGURE 1. Fractional precipitation of BH with ammonium sulfate. The sample was 160 mg. protein containing 180,000 units BH which had been processed through 90° C.-heating of 2% NaCl extract of acetone-dried 23,000 brains. Numerals in parentheses at the top of each histogram indicate the specific activity (*Samia* units/mg. protein).

against 3 changes of 4-liter amounts of 0.05 *M* Tris-HCl, pH 7.8. The addition of ammonium sulfate did not significantly lower the pH of the solution during the above experiment, presumably due to the buffering potency of the extracted substances so that no pH adjustment was necessary.

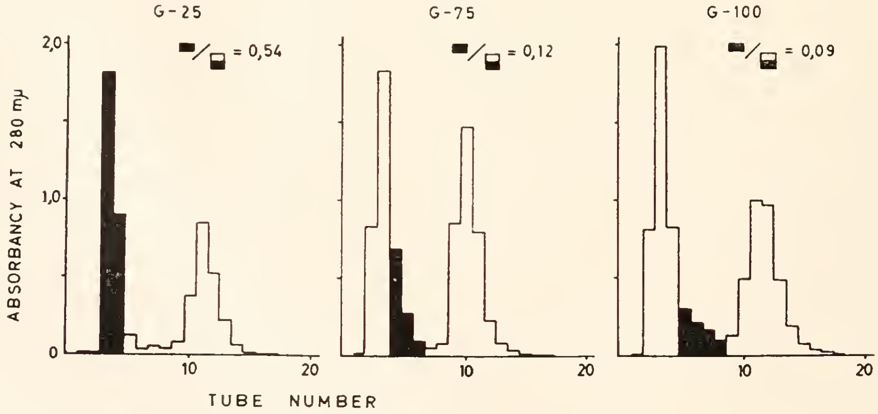

FIGURE 2. Gel-filtration on Sephadex G-25, -75, and -100 columns. Column size, 1.3 × 19.4 cm. Elution buffer, 0.05 *M* Tris-HCl, pH 7.8. Flow rate, 12 ml./hr. Each fraction, 3 ml. The sample for each column was 10 mg. protein containing 40,000 units BH, an aliquot of 30–65% fraction represented in Figure 1. Solid bars represent fractions which contained more than 150 units BH.

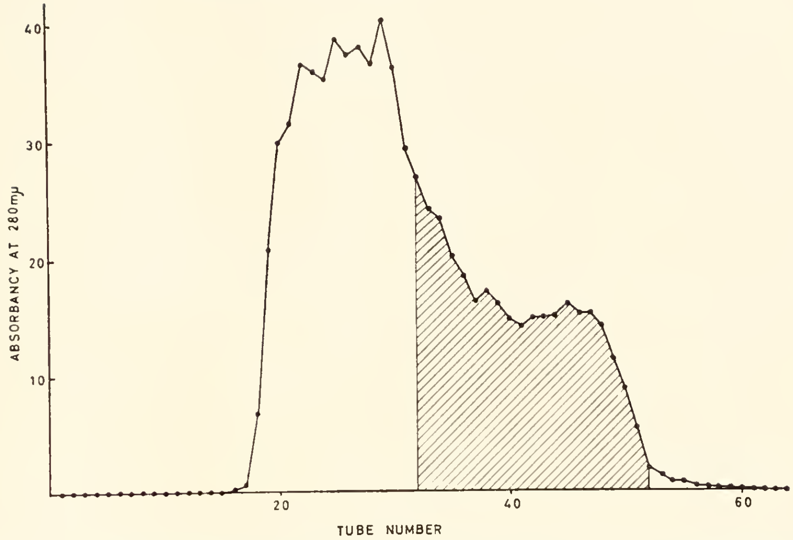

FIGURE 3. First gel-filtration on Sephadex G-100 column. Shaded fractions contained more than 10,000 units BH.

This preparation is designated as *Ammonium Sulfate 30–70% Fraction.* It contained 1,120,000 units BH; the slight rise in total BH units from the preceding step is not significant.

First gel-filtration on Sephadex G-100 column. Preliminary experiments of gel-filtration on Sephadex columns were performed using 3 types of Sephadex, namely G-25, G-75, and G-100. The results are shown in Figure 2. BH was completely excluded from the particles of G-25 whereas partially excluded from G-75 and -100. It is apparent that G-100 is most effective for separation of BH.

The *Ammonium Sulfate 30–70% Fraction* was then subjected to gel-filtration on a 3.7 × 37.0 cm. column of Sephadex G-100. The elution was with 0.05 *M* Tris-HCl, pH 7.8. The flow rate was 60 ml./hr. and 10-ml. fractions were collected. Aliquots from each of the fractions were diluted 20 times with the buffer, and assayed by injecting 0.02 ml. into each of assay pupae. By this assay one can know that fractions which turn out positive must contain more than 10,000 units BH while negative fractions contain less than 10,000. The results are shown in Figure 3. The fractions 32–52 turned out positive. They were pooled and subjected to further purification. This pooled fraction is designated as *First Sephadex G-100 Fraction.*

Stepwise chromatography on DEAE-cellulose. Preliminary experiments indicated that BH bound to DEAE-cellulose at low molarity of Tris-HCl, pH 7.8, while it was eluted with the same buffer of 0.5 *M*. CM-cellulose failed to absorb BH, when applied in 0.01 *M* Tris-HCl, pH 6.5.

The *First Sephadex G-100 Fraction* was subjected to stepwise chromatography on a 2.8 × 36.0 cm. column of DEAE-cellulose. The stepwise elution was performed using 0.05 *M,* 0.14 *M,* 0.3 *M,* and 0.5 *M* of Tris-HCl, pH 7.8, and 0.5 *M* of this buffer containing 0.1 *M* NaCl. The flow rate was 35 ml./hr. and 7-ml. fractions

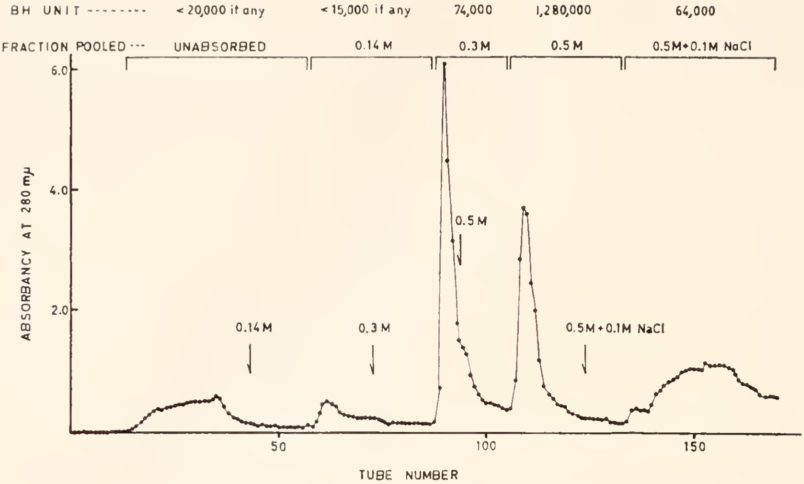

FIGURE 4. Stepwise chromatography on DEAE-cellulose. Tris-HCl buffers (pH 7.8) of the specified molarities were applied at the places designated by arrows. Brackets represent the pooled fractions.

were collected. The effluents at each step were pooled and BH units contained in them were determined. The results are shown in Figure 4. The most active fraction was the 0.5 *M* fraction which contained 1,280,000 units BH. The 0.3 *M* fraction and 0.5 *M* + 0.1 *M* NaCl fraction contained 74,000 and 64,000 units, respectively. The unabsorbed fraction and 0.14 *M* fraction turned out negative when tested by injecting 0.02 ml. into each assay pupa, indicating that they contain less than 20,000 and 15,000 units, respectively. The 0.5 *M* fraction was subjected to subsequent purification and is designated as *DEAE-Cellulose 0.5 M Fraction.*

Gradient chromatography on DEAE-cellulose. The *DEAE-cellulose 0.5 M Fraction* was dialyzed against 0.25 *M* Tris-HCl, pH 7.8, and placed on a 1.9 × 30.0 cm. column of DEAE-cellulose equilibrated with the above buffer. The column was washed with the buffer and gradient chromatography carried out between 300 ml. each of 0.3 *M* and 0.5 *M* Tris-HCl, pH 7.8. The flow rate was 35 ml./hr. and 8-ml. fractions were collected.

Because of the limited number of assay pupae available, the bioassay of 75 fractions was carried out in two steps as follows. First, aliquots from all fractions were diluted 20 times with deionized water, and assayed by injecting 0.02 ml. into each of the assay pupae. By this assay one can distinguish fractions which contain more than 8,000 units BH by their positive responses. After knowing the result of the first assay, the positive fractions only were again assayed to determine the actual amount of BH.

The result is shown in Figure 5. Two discrete groups of BH activity, tubes 6–16 and 23–27, are seen. In addition, the profile of BH activity within each of these groups is irregular, suggesting a high heterogeneity in the molecular form of BH. It should be added further that the washing with 0.25 *M* buffer contained 80,000 units BH. The tubes 6–16 were pooled and only this fraction was subjected

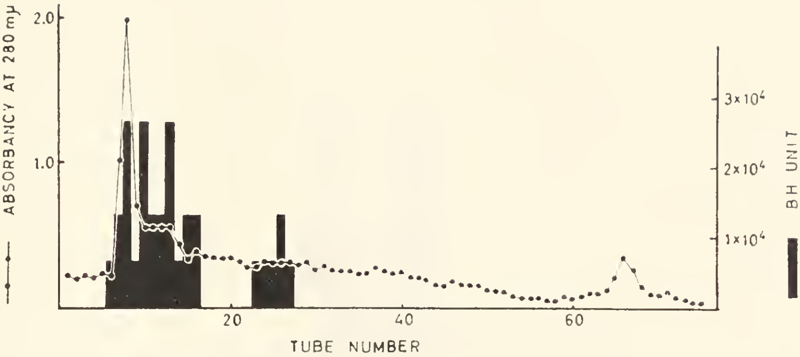

FIGURE 5. Gradient chromatography on DEAE-cellulose.

to further purification. This fraction is designated as *DEAE-Cellulose 6–16 Fraction.*

One may notice that the total activity of BH illustrated in Figure 5 (248,000 units) plus the activity in the washing (80,000 units) is much smaller than that of the starting material in this chromatography (1,280,000 units). However, the first assay employed here implies a possibility that each of the negative fractions might have contained up to 4000 units BH. If all of the 59 negative fractions had contained 4000 units, then $4000 \times 59 = 236{,}000$ units could have been present in these fractions. A sum of all of the above is 564,000. In view of the experimental error necessarily involved in the assay method using 2-fold-dilutions, the difference between 1,280,000 and 564,000 units is in the range of the experimental error. But the above calculation is based upon the assumption that the maximum possible amounts were present in all of the negative fractions. The actual amount in the negative fractions is possibly much smaller: in that case the total amount of BH recovered is too small to ascribe to the experimental error. It is possible that partial inactivation of BH occurred in this purification step.

Second gel-filtration on Sephadex G-100 column. The *DEAE-Cellulose 6–16 Fraction* was precipitated with ammonium sulfate, with pH adjustment at 6.4–6.8 with 0.1 N NaOH. The precipitate was dissolved in 3 ml. of 0.05 *M* Tris-HCl, pH 7.2, and dialyzed against the same buffer. This solution was again subjected to gel-filtration on a 2.8 × 32.0 cm. column of Sephadex G-100. The elution was performed with the above buffer at a flow rate of 30 ml./hr. Fractions were collected each having a volume of 9 ml. Bioassay was again carried out in two steps. In the first step all of the 32 fractions were assayed by injecting 0.02 ml. into each assay pupa. Tubes 4–28 were positive, indicating that they contained more than 450 units BH. The actual amounts of BH were determined only for these positive fractions. The result is shown in Figure 6. The profile of BH activity again suggested the presence of highly heterogeneous components of BH. Tubes 4–16, 17–21, and 22–28 were pooled separately and designated as *Second Sephadex G-100 Fraction-I, -II,* and *-III,* respectively. It is apparent that all of these fractions were still accompanied by considerable amounts of other materials,

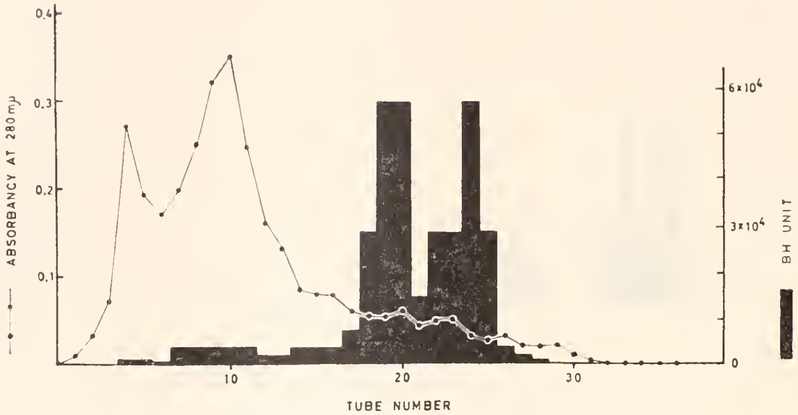

Figure 6. Second gel-filtration on Sephadex G-100 column.

in view of lack of correspondence between the profile of BH activity and optical density at 280 mμ.

2. *Estimation of approximate molecular weights of the Second Sephadex Fractions*

The elution of proteins from Sephadex columns is correlated with their Stokes radii and, if the molecules are globular, with their molecular weights (Siegel and Monty, 1966). Based upon this fact the approximate molecular weights of impure preparations of proteins can be estimated by means of Sephadex gel-filtration (Andrews, 1964; Siegel and Monty, 1966).

The molecular weights of the *Second Sephadex G-100 Fraction-I, -II,* and *-III* were estimated by this method. The volume of each of the *Second Sephadex Fractions* was reduced to 2 ml. by means of ammonium sulfate precipitation and then used. The substances of known molecular weights used as standard were blue dextran (Pharmacia, M.W., 2,000,000), γ-globulin (Fraction 2 from bovine plasma, Armour, M.W., 180,000), ovalbumin (Merck, M.W., 45,000), pepsin (Merck, M.W., 35,000), α-chymotrypsin (M.W., 22,500), yeast cytochrome *c* (M.W., 12,950), and bromphenol blue (M.W., 670).

A 3.7 × 35.0 cm. column of Sephadex G-100 was used. The elution was performed with 0.03 *M* Tris-HCl, pH 7.6, at a flow rate of 30 ml./hr. Fractions were collected, each with a volume of 7 ml. Special care was taken to prepare a column which gave good reproducibility on separate runs. To this end, the Sephadex was allowed to swell with deionized water for at least 7 days; it was then packed in the column and the elution buffer was continuously run for 4 days before use. The BH fractions and the standard molecules (10 mg. protein in 1.5 ml. buffer) were separately run and the proteins were read by optical density at 280 mμ, except for cytochrome *c* which was read at 550 mμ. Blue dextran and bromphenol blue were read at 370 mμ. The results were combined and shown in Figure 7. One may notice that the lower molecular-weight limit for complete exclusion of proteins from Sephadex G-100 in the present result is much smaller than that listed in the literature. This may be due either to the difference in lot

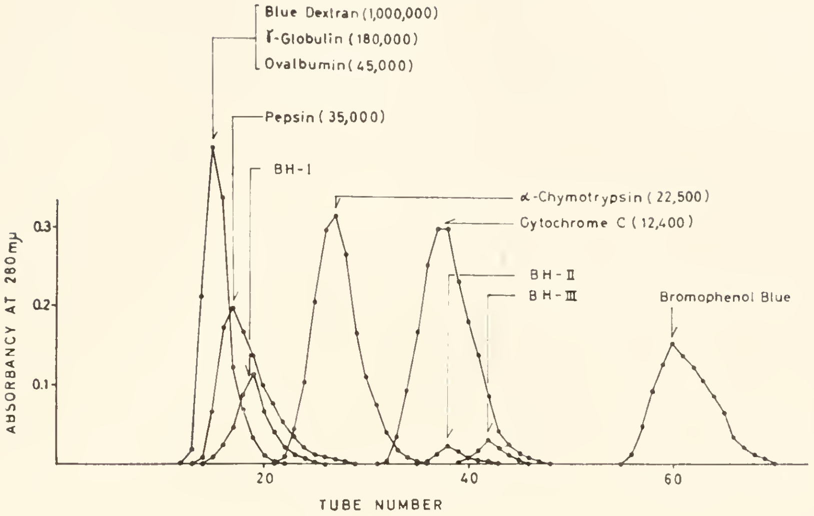

FIGURE 7. Gel-filtration on Sephadex G-100 column of the *Second Sephadex G-100 Fractions* and other substances of known molecular weights. Blue dextran, bromphenol blue, and cytochrome *c* were measured at the wave-lengths specified in text. Blue dextran, γ-globulin, and ovalbumin exhibited similar curves so that only plotting of γ-globulin was presented. BH-I, -II, and -III indicate the *Second Sephadex G-100 Fraction-I, -II,* and *-III,* respectively.

number of Sephadex used or to a slight difference in procedures. In any event, the elution pattern was satisfactorily reproducible between separate runs of identical materials, so that there must have been no defect on the estimation of the molecular weights. The approximate molecular weights of BH were thus estimated as 31,000, 12,000 and 9000 for the *Second Sephadex Fraction-I, -II,* and *-III,* respectively.

DISCUSSION

About 8000-fold purification of BH was accomplished and only 0.002 μg. of the most purified preparation, the *Second Sephadex G-100 Fraction-II,* as determined by protein measurement, was active to cause adult development in a *Samia* assay pupa. The data suggested, however, that the most purified preparations were still accompanied with too many other substances.

The purification procedures employed in this study were all those used routinely for protein purification and BH was successfully purified by these procedures. On the basis of this fact and inactivation of BH by some proteolytic enzymes (Ichikawa and Ishizaki, 1963), we assume that BH is a polypeptide(s) or small protein(s). Recently Kobayashi and Yamazaki (1966) obtained similar results on the proteinaceous nature of BH.

Evidence for the proteinaceous nature of the neurosecretory substances in invertebrates has increasingly been accumulated, conforming to the well established fact that the known neurosecretory substances in vertebrates are polypeptides. Among them are a tanning hormone ("bursicon") in the fly and cockroach brain

(Fraenkel and Hsiao, 1963, 1965; Fraenkel *et al.*, 1966; Mills and Lake, 1966), hyperglycemic and heart-beat-accelerating hormones in the cockroach corpus cardiacum (Davey, 1961; Steele, 1963; Natalizi and Frontali, 1966), heart and hindgut activating hormones in the cockroach corpus cardiacum (Brown, 1965), light-adapting distal retinal pigment hormone and erythrophore-dispersing and -concentrating hormones in the crustacean eye-stalk (Josefsson and Kleinholz, 1964; Kleinholz and Kimball, 1965), and gamete-shedding hormone in the starfish radial nerves (Kanatani and Noumura, 1962, 1964; Chaet, 1964, 1966). The present study on BH adds to the evidence generalizing that the neurosecretory substances are polypeptides or proteins in invertebrates also.

The BH activity manifested highly heterogeneous profiles in DEAE-cellulose chromatography as well as in Sephadex gel-filtration. The heterogeneity in the electric charge as well as in the molecular weight is thus presumed to exist in the molecular form of BH. Three alternative explanations for this heterogeneity are possible: (1) BH itself has multiple molecular forms as in the case of the isozymes or hemoglobin, (2) BH is a single molecule but is associated with other proteins resulting in an apparent heterogeneity, (3) heterogeneity is due to chemical modifications occurring during the purification procedures. But, at present, it cannot be decided which explanation is true.

Kobayashi and Yamazaki (1966) have reported that BH is bound to CM-cellulose but not to DEAE-cellulose. This is precisely the reverse of our results, notwithstanding the use of the same extraction material, *Bombyx* brains and the use of similar buffers. This difference is apparently due to methodology, for the above mentioned investigators did not expose their crude extract to heat or to ammonium sulfate precipitation prior to chromatography.

Kobayashi and his associates (Kobayashi *et al.*, 1962a, 1962b; Kirimura *et al.*, 1962; Saito *et al.*, 1963) have earlier purified an oily substance which can activate the prothoracic glands and identified it as cholesterol. Since then, they have concluded that cholesterol is one of BH, the other being a polypeptide (Kobayashi and Yamazaki, 1966). However, as pointed out by Schneiderman and Gilbert (1964), cholesterol is normally present in the blood at a titer 10,000 times higher than that which is able to cause adult development in the assay pupa when introduced by injection. On the basis of well-documented data concerning the prothoracotrophic activity of the juvenile hormone-mimicking substances, Krishnakumaran and Schneiderman (1965) considered that cholesterol activates the prothoracic glands not because it resembles BH but because it resembles juvenile hormone or ecdysone.

"Neurohormone D_1" or "activation hormone" was obtained in a crystalline form by Gersch and his associates (Gersch *et al.*, 1960) and was proved to activate the ring gland of *Calliphora erythrocephala* (Gersch, 1961, 1962). Reports on the characterization of their substance are awaited. It is also hoped the biological assays with other test animals will be done. The *Calliphora*-test seems inappropriate for the assay of BH, since the dipteran brain-ring gland interaction involves an exceptionally high degree of the neural control (Possompès, 1950, 1958). This may have led to rather ambiguous results in the case of neurohormone D_1.

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SUMMARY

1. The brain hormone extracted from brains of *Bombyx mori* was purified by use of heating, ammonium sulfate precipitation, Sephadex gel-filtration, and DEAE-cellulose chromatography. On the basis of protein measurement, about 8000-fold purification was achieved. The most purified preparation was active by 0.002 μg. protein when injected into brainless pupae of *Samia cynthia ricini*.

2. The brain hormone manifested highly heterogeneous molecular forms which were revealed by Sephadex gel-filtration and by DEAE-cellulose chromatography. The molecular weights of the major components were estimated by Sephadex gel-filtration as ranging from 9000 to 31,000.

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FEEDING AND OVIPOSITION BEHAVIOR IN THE MOSQUITO AEDES AEGYPTI (L.). I. PRELIMINARY STUDIES OF PHYSIOLOGICAL CONTROL MECHANISMS

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Fundamental behavioral patterns associated with feeding and egg-laying have long attracted the attention of biologists, although among insects little is known of the physiological mechanisms controlling these activities. Mosquitoes offer a particularly advantageous organism in which to study these processes, since feeding and oviposition are well-defined endpoints, and because a considerable fund of knowledge on the relation between feeding and ovarian development exists.

The cyclic nature of biting and oviposition by females of *Aedes aegypti* has been recognized for some time (MacFie, 1915; MacGregor, 1931). Lavoipierre (1958), however, was the first to point out the presence of two distinct biting patterns in this species, and demonstrated that these patterns were determined by whether the female was virgin or had mated. A second behavior pattern, again depending on whether or not insemination had occurred, is noted when oviposition is studied. Virgin females, while maturing the same number of eggs following a blood meal as do mated females, retain all, or most, of the eggs. These eggs will be deposited following mating, but in the absence of mating will be retained until the death of the female. Mated females, on the other hand, oviposit readily and completely on completion of oogenesis, provided a suitable site is available. This phenomenon was first reported by both MacFie and MacGregor. Further documentation was provided by Gillett (1955), Lang (1956), Wallis and Lang (1956) and Lavoipierre (1958). In contrast, Gillett also reported a single West African strain of this species in which no difference exists in the oviposition behavior of mated and virgin females, both laying their eggs on maturation.

With this single exception, it is well documented that differences in behavior between mated and virgin females of *A. aegypti* do exist, and that the virgin patterns can be changed to the mated type by some aspect of the mating act. A "system" thus exists which is well suited for investigating the physiological mechanisms by which these patterns can be altered. This paper reports the preliminary findings of this investigation.

METHODS

Mosquitoes of the Liverpool strain of *A. aegypti* which had been cultured in this laboratory for several years were used in this study. To reduce variability, a standard rearing procedure was adapted. Two hundred fifty newly hatched larvae were placed in 500 ml. of water in an 11″ × 7″ × 2″ deep enamel pan. Finely ground cattle food (Misco Mills High Protein Supplement No. 2) was provided as food, 0.60 g. being added per pan on days 2 and 5 (day of hatching equals day 1), and 1.20 g. on day 7. Preliminary tests showed this to be an excess amount of food for this number of larvae, so that the carryover of nutrients into the adult stage was equalized between batches of mosquitoes.

Pupae were sexed and then isolated in small cages. On emergence adult females were randomly collected and transferred to foot-square plywood and Plexiglas (top and one side) cages. Water and a cube of sucrose were continuously available. When mated females were required, a surplus of males was added to the cage, left for 24 hours, and the males removed at least 24 hours prior to the beginning of feeding tests. Preliminary experiments consisting of examination of ovaries for the presence of mature eggs and the spermathecae demonstrated that all females were inseminated under these conditions.

Females were allowed to engorge on a human hand; non-feeders were removed from the cage so that the population used in these experiments consisted wholly of females which fed during the first opportunity. On subsequent days a 10-minute feeding opportunity was offered this group, and the numbers ingesting blood recorded. Feedings tests were conducted between 9:00 and 10:00 AM each day, to avoid any variation due to a feeding rhythm even though none has been demonstrated in this strain (Khan *et al.*, 1965), and the females always allowed to feed to repletion. Oviposition behavior was determined by continuously providing a small glass or waxed-paper dish containing distilled water and a strip of paper towelling. Egg counts were made daily following the feeding tests.

The possibility that stimuli arising from physical union during copulation were responsible for inducing the mated type of behavior was tested by allowing virgin females to copulate with "depleted" males in the manner of Gillett (1955). These males had previously been allowed to copulate in a separate cage with a minimum of 6 females in rapid succession, and had generally depleted their supply of semen. The test females were introduced singly into a cage containing a few "depleted" males, and were removed immediately following a single union. Microscopic examination of the spermathecae following the tests verified that few or no sperm had been transferred during these matings.

The semen transferred during copulation constitutes another factor which might have been responsible for the "mated effect." Any effects of the components of this fluid were determined by introducing an isolated testis or lobe of the symmetrically bilobed accessory gland into newly emerged virgin females and determining the resulting patterns of biting and oviposition. Females were immobilized by chilling and placed in a restraining device (Lavoipierre and Judson, 1965). The organ under investigation was taken up in a saline-filled micro-pipette and injected into the thorax through a slit in the metapleuron.

The possible participation of the ovaries in the regulation of biting behavior was investigated by implanting a normal ovary into females in which development of these organs had been inhibited. The chemosterilant Apholate (Olin Mathieson Chemical Corp.), added to larval pans (day 2) at a rate of 25–30 ppm interfered with development of the ovary. Only a rudimentary non-functional organ was present in emerging females. An ovary from a normal untreated female was injected into the thorax of these females, and then the female was mated or received an implant when required, and the biting pattern determined. All implants and injections were made using the saline of Hayes (1953), and the females allowed a 36–48-hour recovery period prior to beginning the feeding tests. Feeding or

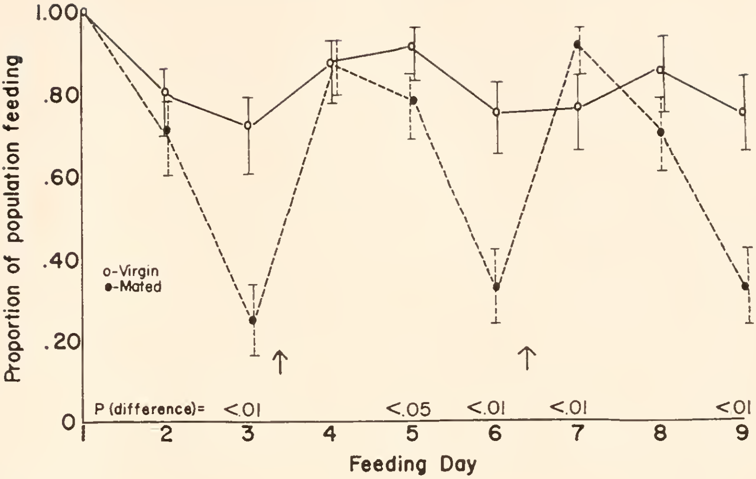

FIGURE 1. Feeding patterns of mated and virgin female *Aedes aegypti,* 95% confidence limits. Arrows denote oviposition by mated females.

biting as used here refers to the actual ingestion of blood. Careful observation during these tests demonstrated that females of this strain did not probe or penetrate the host's skin without ingesting blood, and in fact, any female which was "activated" (Laarman, 1958) to leave the cage wall and alight on the host invariably took blood. The amount of blood ingested varied due to the presence of residues of previous meals in the gut, as well as with the presence or absence of eggs in the ovaries, although some definite expansion of the abdomen always occurred. All rearings and tests were carried out at 80 ± 4° F. The relative humidity of the rearing room was 75 ± 5%, but within the cages was somewhat higher.

RESULTS

The pronounced alteration of the feeding pattern of virgin *A. aegypti* which is induced by mating can be seen in Figure 1. While at least 75% of the virgin female population fed daily, the proportion of mated females feeding on days 3, 6, and 9 was significantly reduced ($p < .01$). These periods of low feeding avidity occur during the terminal portions of the first, second, and third gonotrophic cycles, respectively. Oviposition by mated females (see below) occurs within hours (6–18) of these low points; on the following day eggs had been laid, avidity for a blood meal had increased markedly, and the subsequent gonotrophic cycle initiated. The feeding behavior of virgin females is also cyclic, but weakly so [significantly more females fed on days 1 and 5 than on day 3 ($p = .05$)]. This cycle shows a 4- to 5-day period rather than the precise 4-day period of the mated females.

Since these results clearly indicate that some aspect of the mating act is responsible for modifying the virgin biting and oviposition patterns, tests were conducted to identify the factor(s) responsible, using the proportion of the test population feeding on day 3 or the total number of bites over a 3-day period, as

TABLE I

The effect of repeated matings by males on semen depletion and biting and oviposition by female Aedes aegypti

	No. of copulations by male			Control	
	1–3	4–6	7 plus	Virgin	Mated
No. females/group	21	16	26	12	17
Total bites, days 1–3	41	36	60	31	28
Average bites/female, 3 days	1.9	2.3	2.3	2.6	1.6
Total eggs laid, days 4–6	813	512	234	106	967
Average eggs laid/female	39	36	9	9	57
Per cent egg hatch	80	65	64	0	94
Per cent females inseminated*	75	69	20	—	—

* By examination of spermathecae.

the basis for comparison. Use of the technique of repetitive matings by single males, with consequent depletion of semen, demonstrated that the "mated effect" was not a result of the physical union during copulation (Table I). Here the expression of the mated type of biting and ovipositional behavior patterns by the females declines with the male's ability to transfer semen successfully, but not with his ability to unite with the female. These results indicate the likelihood that the seminal fluid is the effective agent in modifying these behavioral patterns.

Implantation of the individual organs contributing to the seminal fluid into virgin females demonstrated that the presence of an accessory gland lobe, but not a testis, induced a biting pattern with all the characteristics of normally mated females (Table II). The proportion of the virgin female population which had received a testicular implant, and which fed on day 3, did not differ (65%) from that of the sham-operated (saline-injected) virgins (64%). In contrast, females receiving male accessory gland showed significantly less ($p < .01$) avidity on feeding day 3 (24%) than did either testicular-implanted females (65%) or sham-operated virgins (64%).

While these experiments indicated that alteration of the virgin biting pattern

TABLE II

Effect of implanted male organs in modifying feeding behavior of virgin female Aedes aegypti

Implant	No. feeding	Virgin female feeding behavior; day 3.	
		Mean, per cent	(95% Confidence limits)
Accessory Gland	11/46	24*	(12–39)
Testis	31/48	65	(49–78)
Virgin operated control	27/42	64	(47–79)
Mated operated control	11/29	38	(18–67)

* Significantly less ($p = < .01$) than testis or virgin operated control.

TABLE III

*Apholate-induced inhibition of ovarian development and the biting behavior of female Aedes aegypti**

Condition		Number	Females feeding, Day 3	
Female	Male		Per cent	(95% Confidence limits)
A.				
Treated-virgin control		132/167	79	(70–86)
Normal-virgin control		69/96	72	(62–81)
Treated × normal		32/48	67	(52–81)
Normal × normal		28/111	25	(17–34)**
B.				
Treated × normal-operated control		13/16	81	(53–96)
Treated × normal + implanted ovary		2/9	22	(02–59)**

* Exposed to 25–30 ppm Apholate as larvae.

** Proportion of females feeding is significantly less ($p = .02$) than other females within the group.

is brought about by male accessory gland material, an additional component of the control mechanism is indicated by the demonstration that the ovaries of the recipient female also participate in the regulation of this activity. As shown in Table IIIA, biting by Apholate-treated females is always of the "virgin" type, even when these females had mated with normal (untreated) males. In these cases, the absence of a functional ovary abolishes the expected action of the male accessory gland. When these treated females received an implanted normal ovary, however, the expected effect of the accessory gland material (or mating) in altering the virgin pattern is restored (Table IIIB).

A second behavioral characteristic of mated females, which contrasts with the behavior of virgin females, is the readiness of the former to deposit newly matured eggs. The thoracic injection of accessory gland material into virgins caused them to oviposit readily on completion of oogenesis (Table IV). In addition, the proportion of the implanted female population ovipositing is similar to that of the normally mated females. Implantation of a testis did not produce a comparable effect. Dissections of both mated and virgin females during the present experiments demonstrated (Table IV) that equal numbers of eggs were produced by the females regardless of the state of insemination.

DISCUSSION

The distinctness of the biting patterns shown by virgin and mated females of *A. aegypti* has been demonstrated by these experiments and verifies the results of Lavoipierre (1958). The mechanism responsible for modifying the virgin pattern into that of the mated pattern is as yet unknown, but these experiments demonstrate that it is triggered by the accessory gland component of the seminal fluid transferred to the female during copulation. Corollary experiments have demonstrated that no chemical stimulus originating in the testis, nor stimuli associated with either physical union during copulation or the transfer of semen through the female's

TABLE IV

Oviposition by virgin female Aedes aegypti receiving implants of testis or accessory gland

	Implant		Sham-operated	
	Testis	Accessory gland	Virgin	Mated
A. Number of eggs/no. of females:				
Eggs laid	711/27	1905/25	542/31	869/12
Mean/♀♀	26	76	18	72
Eggs retained*	1430/27	292/20	1930/28	143/10
Mean/♀♀	53	15	69	14
Total eggs formed Mean/♀♀	79	91	87	96
B. Proportion of test population ovipositing*				
Number	10/27	18/21	6/28	8/10
Per cent	37	86	21	80
(Range)	(13–47)	(75–100)	(0–46)	(75–100)

* Determined by dissection of ovaries: oviposition was considered to have occurred if ovaries contained only a few (<20) mature eggs. Differences in numbers of females due to deaths and escapes.

reproductive tract are effective in inducing this response. When matings are conducted between individual virgin females and sequentially mated ("depleted") males, the results indicate that the male's ability to transfer semen falls off rapidly after 4 to 6 matings. Virgin females allowed to copulate with these males continued to show a "virgin" biting pattern despite the fact that the pair remained *in copulo* for a normal length of time. The ineffectiveness of the physical union *per se* in triggering the mated response is thus demonstrated.

In addition, the thoracic location of the implanted accessory gland rules out possible stimuli associated with sperm transfer within the female's reproductive tract, as occurs in *Cimex lectularis* (Davis, 1965), as an effective stimulus in the alteration of this behavior pattern. The location of the effective implant does, however, point to some diffusible chemical as responsible for the observed changes.

In some insects oogenesis is initiated by the mating act (Davis, 1965; Highnam, 1962; Roth and Stay, 1961), so that changes in feeding behavior might be expected to reflect the new requirements of the activated ovaries. Such a correlation between the state of oocyte development and the activity of the corpus allatum and the preferential ingestion of either protein or carbohydrate seems to occur in *Calliphora erythrocephala* (Strangways-Dixon, 1961) and in *Phormia regina* (Dethier, 1961). Such is not the case, however, in mosquitoes where oogenesis is usually initiated by the ingestion of a blood meal and is independent of whether the female has been inseminated or not (Gillett, 1958; Larsen, 1958; Larsen and Bodenstein, 1959). Neither the pronounced cyclic nature of the mated feeding pattern, nor the difference between mated and virgin female biting patterns, is therefore a consequence of an activated ovary *per se*, or any difference in the numbers of eggs being formed. The suppression of the feeding drive in mated females during the terminal phases of each gonotrophic cycle thus seems clearly to be a consequence of the presence of the accessory gland material acting in an unknown manner.

Observations of females during the feeding trials indicate that the lowered avidity on day 2, and especially on day 3, is due to the failure of the females to become "activated" (in the sense of Laarman) and to begin flying and seek out the host, rather than to fly and orient to the host and then failing to alight, probe and feed. One effect of the accessory gland material is therefore an apparent increase in the "threshold of activation," and presumably represents a central nervous system phenomenon.

The prominent increase in the feeding rates of the two types of females on day 4 indicates a "release" of the suppressed avidity characteristic of day 3. This release appears to be brought about by the act of oviposition, but other studies indicate this is not the major controlling factor. An increase in feeding rate from 25 to 90% occurs between days 3 and 4 in mated populations. In virgin females the increase is from 70 to 90%, while in mated females which were prevented from ovipositing (unpublished observations), the rate increased from 25 to 70%. A comparison of these feeding rates between virgin and mated females on the one hand, and between ovipositing and non-ovipositing mated females on the other, suggests that the release of suppressed avidity is not due mainly to the oviposition act, but is a consequence of both the completion of oogenesis and the inseminated condition. Thus the release occurring in non-ovipositing mated females amounted to about 70% of that occurring in mated females allowed to oviposit.

Despite the role of male accessory gland material in suppressing the biting drive during the terminal phases of each gonotrophic cycle, further experiments utilizing Apholate-treated females demonstrated that this is not a singular effect, but is expressed only in the presence of a functional ovary. Since the effects of Apholate are undoubtedly broader than just the inhibition of ovarian development, the possibility of "side-effects" and their participation in the abolition of the accessory gland effects exists. This possibility cannot be completely ruled out in the absence of more detailed knowledge of the mode of action of this material. The fact, however, that a normal ovary, when implanted into a treated female, was able to undergo oogenesis, indicates that the nutritional and endocrine environment within the female had not been significantly altered by the treatment. When, in addition, implantation of male accessory glands or mating, re-establishes the feeding pattern seen in non-treated females, the conclusion that the mechanism controlling the biting drive involves both accessory gland material and the ovary seems justified.

Stimulation of oviposition is also a consequence of the presence of accessory gland material. Virgin females bearing an implanted accessory gland oviposited in a manner similar to mated females in regard to the number of eggs laid per female as well as the proportion of the population depositing their eggs (Table IV). These results substantiate the recent findings of Leahy and Craig (1965). They further extend our knowledge of the control of oviposition in *A. aegypti* by demonstrating the ineffectiveness of testicular material in influencing this behavior (Table IV). Matings between virgins and depleted males are also ineffective in increasing the oviposition rate (Table I), thus verifying Gillett's earlier claim (1955) that a component of seminal fluid rather than than physical stimuli induces this behavior.

Little is known of the characteristics of the male accessory gland substance or the factors controlling oviposition in mosquitoes. Lum (1961) had described the gland secretion of several species as a viscous fluid containing granules. Significantly, he points out that within about 24 hours of mating the granular material has dissolved and largely disappeared from the female's reproductive tract. In *Drosophila melanogaster* the analogous paragonial fluid contains a prominent peptide component (Chen and Diem, 1961). Preliminary tests in the present work indicate that the effectiveness of the gland substance in effecting changes in both biting and oviposition behavior is abolished when the material is boiled for 5 minutes prior to injection. Curtin and Jones (1961) have studied the processes of ovulation and oviposition in *A. aegypti.* Their results suggest that the initiation and coordination of these activities are under neural control with cephalic and thoracic regulatory centers. The mechanism coupling the accessory gland material with this neural mechanism remains to be discovered.

SUMMARY

1. When female *Aedes aegypti* were given a daily opportunity to take a blood meal over a 9-day period, the number of mated females feeding on days 3, 6, and 9 was significantly less than the number of virgins feeding. This low period of feeding coincides with the terminal phases of the first, second, and third gonotrophic cycles, respectively.

2. The "mated" feeding pattern could be established in a virgin female by the implantation of a male accessory gland. Copulation without semen transfer or the implantation of a testis was ineffective in inducing the pattern of biting.

3. Functional ovaries in the female receiving the accessory gland implant are required in order for the implant to exert its effect. Implantation of a normal ovary plus an accessory gland into females lacking ovaries produced the mated biting pattern.

4. The material from the male accessory gland also controls oviposition behavior by female mosquitoes. Mated females lay their eggs readily on completion of oogenesis. Virgins mature an equal number of eggs but retain rather than deposit them. Implantation of male accessory gland into virgin females causes them to oviposit on maturation of their eggs.

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OXYGEN BALANCE IN SOME REEF CORALS[1]

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Ecologists have been intrigued by the seeming paradox of the biological richness of coral reefs in the middle of relatively barren tropical seas. It has been debated whether such a community lives mostly off material in the water passing over it or whether it produces at least part of its required fixed carbon by local photosynthesis (Yonge *et al.*, 1932; Odum and Odum, 1955; Ryther, 1959). The latter thesis is suggested by the large numbers of unicellular algae within the living cells of the coral and the boring filamentous algae in the non-living skeleton of the coral.

Others have shown that under certain experimental conditions, a coral head may produce more oxygen by photosynthesis than it consumes by respiration (Yonge *et al.*, 1932; Kawaguti, 1937; Odum and Odum, 1955; Burkholder and Burkholder, 1960; Beyers, 1963 and 1966; Franzisket, 1964; Roffman, personal communication). We have extended this work by continuously monitoring oxygen tension in the experimental chamber at different light intensities and by relating oxygen tension to the area of the coral head receiving light. The coral heads we used include the coral animals, their intracellular dinoflagellates (zooxanthellae) plus, in stony corals, the filamentous green algae residing inside the non-living $CaCO_3$ skeleton, and any number of animal species and micro-organisms that may also inhabit the skeleton. From such measurements we find isolated coral heads to be nearly as productive as any other organisms in nature, yielding values up to 10 grams carbon fixed per square meter of reef per day. In addition our data indicate that most of the photosynthesis is that of the zooxanthellae. Whether this productivity actually nourishes the coral animals will only be determined by tracer experiments and by quantitative observations of corals with and without zooxanthellae.

MATERIALS

Oxygen balance experiments were carried out on the following species of corals:

Octocorallia, Gorgonacea:
    Plexaura flexuosa Lamouroux
    Gorgonia ventalina L.
    Briareum asbestinum (Pallas)
    Erythropodium caribaeorum (Duchassaing & Michelotti)

Zoantharia, Scleractinia
    Siderastrea siderea (Ellis & Solander)
    Porites divaricata Lesueur

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[1] Contribution No. 1982 from the Woods Hole Oceanographic Institution.

Favia fragum (Esper)
Manicina areolata (L.)
Montastrea annularis (E. & S.)
Oculina diffusa Lamarck
Dichocoenia stokesii Milne-Edwards & Haime
Mussa angulosa (Pallas)
Isophyllia multiflora Verrill
Colpophyllia sp.

Corals were collected from patch reefs identifiable on U. S. Coast and Geodetic Survey charts of the Florida Keys as "Hen and Chickens" and "The Rocks," both southeast of Plantation Key. Maximum depths at the two sites are 9 m. and 5 m., respectively. Each coral was collected by divers trained to select for size and shape to fit respirometer chambers, for paucity of associated biota, and for ease in collecting and handling. Corals were chipped off the reef with geologists' picks and handled only by their non-living bases so that their tissues were neither touched by hand or tool nor exposed to air in being transferred from reef to laboratory to respirometer. It is important to state that the tissues of stony corals are only four layers of cells thick and that when they are exposed to air or when they are stimulated to contract strongly by being touched in handling, the delicate tissues are perforated in many places by their own $CaCO_3$ skeletons. Experiments performed with animals collected without such care are thus dealing with wounded, presumably regenerating animals. The qualitative and quantitative effects of this treatment on the normal functions of any coral species are unknown and it is therefore desirable to avoid this trauma.

Corals were maintained in sea water in a plastic wading pool. Many more specimens were collected than were used experimentally. Criteria for experimental suitability were the expansion of polyps in the lab at the same time of day the species were observed to be expanded on the reef, and sensitivity of expanded polyps to being jarred or touched that seemed, subjectively, to be similar to sensitivities observed of the species on the reef. Epifaunal organisms were carefully picked off each specimen. In every case but one, corals with polyps expanded were put into the respirometer without being caused to contract completely. All handling was done under sea water. Immediately upon assembly of the respirometer, all specimens expanded their polyps.

We have had little confidence in most previous determinations of coral productivity for a variety of reasons. Foremost is the suspicion that goes with the Winkler O_2 method, used in all previous studies, because of its proneness to chemical interference. Also such before-and-after measurements assume a constancy of rate and thus an independence of the rate of oxygen consumption from the continually changing O_2 tension. Values for respiration have been related to such parameters as the amount of chlorophyll, and thus, whatever their other usefulness, are awkward to employ on an area basis for ecological considerations.

It is now possible to monitor O_2 tension continuously with an electrode (Kanwisher, 1959). This is a physical method immune to chemical interference. We thought it worthwhile to make determinations of productivity of isolated corals using the oxygen electrode in a closed container *in vitro*, with carefully collected corals under measured light intensities, to gain a more accurate notion of photosynthesis in individual coral heads.

METHODS

A. Oxygen measurement

A polarographic oxygen electrode inserted in a chamber with the coral sample continuously monitors the oxygen content of the sea water. Exchange at the free air surface was eliminated by a stopper. A short tube fitting over the electrode contained a stirring magnet supported on a shaft. This was rotated magnetically from outside. It kept the water at the electrode face well stirred (Kanwisher, 1959) and slowly mixed the contents of the chamber without agitating the coral polyps.

The temperature in the experimental chamber ranged from 28 to 31° C. The oxygen exchange at each light intensity was recorded for at least one hour. The electrode current was indicated on a potentiometer recorder from the voltage drop it produced across a thermistor molded in the electrode. The slope of the resulting temperature-compensated oxygen-time curve, combined with the amount of water in the chamber and the solubility coefficient of oxygen, allows the net exchange of the given coral specimen to be computed (Kanwisher, 1966). We then normalize this for the projected area normal to the light beam. Such a figure is equivalent to a rate per unit area of the bottom covered, the most direct way of expressing photosynthesis on the reef. One CO_2 molecule was assumed to be fixed for every O_2 molecule photosynthetically released. The productivity was then calculated as the rate of carbon fixation on an area basis.

The coral in the wide-mouth glass jar was illuminated by varying intensities from incandescent flood lights placed at different distances. The intensities were measured by a waterproof sensor held at the position of the coral surface. A water bath in the light path prevented temperature rise at high light intensities. A sample record is shown in Figure 1. The time constant of the electrode was 15 seconds for 90% response and the mixing time of the chamber was about 30 seconds. This method has been used extensively on sea weeds (Kanwisher, 1966).

Following such measurements on the coral species listed above, another set of measurements was made for a colony of *Dichocoenia stokesii*. The coral tissue was then removed from the colony with a toothbrush and blasts of sea water until inspection with a stereomicroscope revealed no remaining tissue or zooxanthellae. This "cleaned" skeleton with its included boring algae was then reintroduced to the chamber and oxygen measurements were taken in dark and various light regimes.

B. Light measurements

Light was measured for each experimental set-up with a logarithmic light meter calibrated to read footcandles of normal daylight spectral composition. The sensor consists of a network of photoresistive cadmium sulfide cells and fixed resistances such that the over-all resistance changed logarithmically with incident light intensity. A series battery and meter gives a reading proportional to resistance. The wavelength sensitivity curve of the cell plus an overlying filter restricts response to

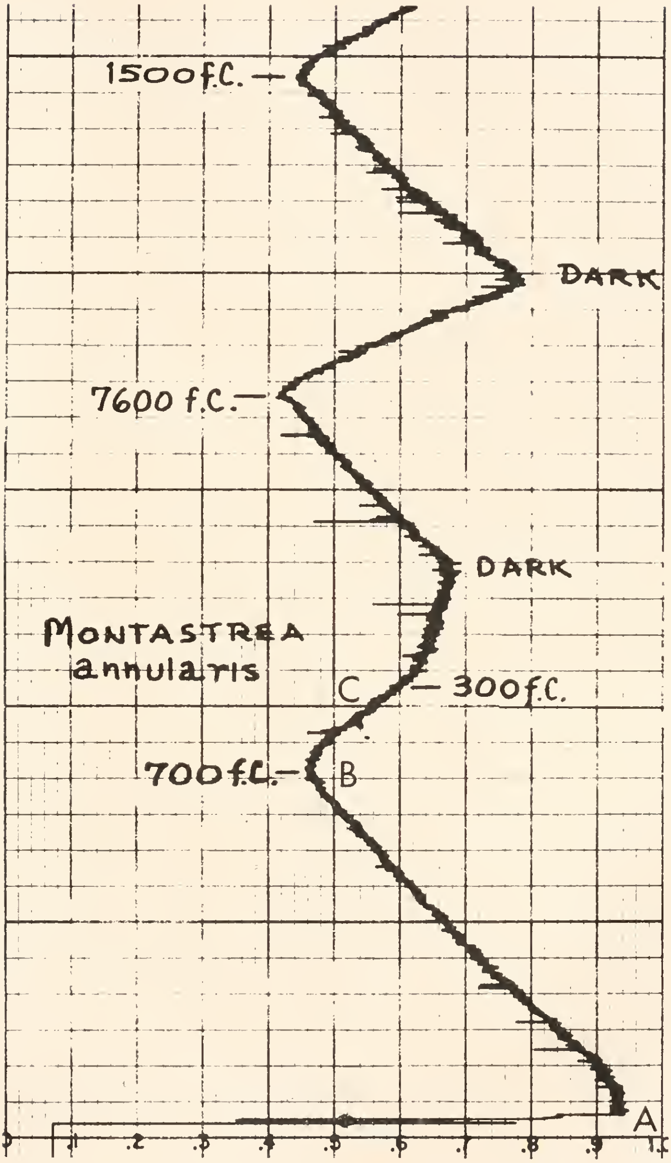

Figure 1. Sample chart record showing the time changes in oxygen produced by respiration and photosynthesis of the coral *Montastrea annularis*. Oxygen content of the sea water increases from 0 on the left to 4.5 ml./liter on the right. From A to B the coral is in the dark and the decrease in oxygen represents respiration. At B the coral is illuminated with 700 footcandles. The resulting photosynthesis is more than twice the dark respiration. For compensation this specimen requires less than the 300 footcandles applied at C. One hour equals 4 units.

wavelengths of 530 to 560 mμ. Since this is in the region of maximum transmission through sea water, the subsurface measurements probably indicate somewhat more total light over the whole visible range than there really is. Light is received over nearly a hemisphere. The same type of meter was used to measure light intensities inside living coral heads under water and through slices of clean coral skeleton under water in the lab.

A $\frac{1}{2}''$ masonry bit in an electric hand-drill was used to bore holes into massive heads of *Montastrea annularis* from the back and from the side. The light sensor (cylindrical: 4 cm. long, 1.2 cm. diameter) could then be inserted into the hole. Light readings were taken in this manner in clean sea water. Corals were kept alive throughout this procedure: drilling was done while the tissues were immersed in sea water. These corals were handled and exposed to air briefly and no expansion of polyps was noted following drilling. Great care was taken not to touch the area of living tissue within 10 cm. of the point directly above the light sensor.

Slices of clean skeleton on *M. annularis* free of macroscopic associated boring organisms were cut 1 cm. thick with a hack saw using new blades that had been cleaned of paint. Cutting was done under flowing water, and packing of dust from the operation into the porous skeleton was thus avoided: sections were found to be clean by inspection with a stereomicroscope. Sections were cut tangentially and radially to the colony, perpendicular and parallel to axes of the calices, respectively. After cleaning the slices, light transmission was measured. The light sensor was held next to the coral slice and stray light from the sensor side was blocked by black cloth.

C. *Boring algae*

Slices, and dissected bits of skeleton, of *Dichocoenia stokesii, Montastrea annularis* and *Mussa angulosa* were examined with a stereomicroscope. Bits of skeleton fixed in 10% neutral formalin in sea water were decalcified under observation in sea water in deep depression slides by adding 3% HCl a drop at a time. The organic remnants were observed up to 1000 × magnification using oil immersion objectives (n.a. 1.3). Thin sections of fixed, dried skeletons were cut and polished to uniform thickness. These were examined with direct microscopy under favorable conditions for the counting and measurement of algal filaments per unit volume of skeleton.

The distribution of boring algae was studied in *Montastrea annularis* by collecting numerous colonies of 10 to 40 cm. diameter, cracking them open along various planes, and observing and photographing the bands of green color. Bits of skeleton from the various green bands were fixed and studied microscopically to determine the state of the algal filaments. Pigment analyses were made on similar material.

RESULTS

A. *Respirometry*

Table I shows maximum figures for gross photosynthesis in grams of fixed carbon per square meter surface area of the bottom per 12-hour day and the

TABLE I

Calculated values of gross photosynthesis per unit surface area, maximum observed ratios of photosynthesis/respiration, and observed or estimated photosynthetic compensation light intensities for some Florida reef corals

Species	Gross photosynthesis q. C/m²./day	Maximum photosynthesis / respiration	Compensation light intensity in footcandles
Gorgonacea			
Plexaura flexuosa	6.5	5.8	400
Gorgonia ventalina	6.8	3.0	300
Briareum asbestinum	3.7	2.5	400
Erythropodium caribaeorum	5.8	4.2	600
Scleractinia			
Siderastrea siderea	4.0	2.1	300
Porites divaricata	10.2	3.4	600
Favia fragum	4.2	2.3	400
Manicina areolata	5.2	2.4	—
Montastrea annularis	9.5	2.9	200
Oculina diffusa	8.4	5.0	—
Dichocoenia stokesii	8.0	—	300
Mussa angulosa	2.7	1.9	300
Isophyllia multiflora	7.9	5.0	400
Colpophyllia sp.	5.4	3.2	700

maximum photosynthesis-to-respiration ratio for each species. Also given is the observed or estimated light intensity in footcandles for photosynthetic compensation. Attention is called to the fact that these figures are not true measures of the performance of algae, but they represent the total respiratory and photosynthetic balance of coral tissue plus zooxanthellae plus other organisms inhabiting the skeleton. Figure 1 shows a typical respirometer record. For any given part of the trace between changes in light intensity, the slope was calculated for the last part of the time in that light regime.

Oxygen production of the boring algae in the "cleaned" skeletons of *Dichocoenia* could not be detected. Oxygen consumption in the dark was measured on both colonies that were treated in this way, but no change in slope of the respirometer trace was detectable following the onset of light intensities of 400 and 5000 footcandles for 1.5 hours.

In our fervor to have polyps expanded we nearly missed seeing the striking effect polyp contraction has in gorgonians. Jarring the respirometer caused *Erythropodium* to contract its polyps during the experiment and external evidence of respiratory exchange all but vanished. We were unable to discern which of two probable causes may have been most important in this apparent inhibition: (1) There is a considerable in- and out-going circulation of sea water created by cilia in the vast system of gastrodermal canals deep inside the colony. When mouths are open this mixing would carry results of gaseous exchange outside the colony very rapidly. When polyps are contracted within the colony, gaseous exchange must be *via* diffusion through the rather dense tissues and would thus be greatly retarded. (2) Mesodermal spicules of calcite are highly refractive to

TABLE II

Light intensity values in footcandles at various depths on the reef

Cloud conditions	Depth m.	Light intensities in footcandles
Bright sun		
9:45–11:30 AM		
over sand	0	4500
	8 on bottom	1000
over coral	0	4500
	4 on bottom	1000
over sand and coral patches	0	4500
	30 on bottom	400
Nearly complete cloud cover		
over sand	0	2000
	5 on bottom	1000
over coral	0	2000
	4 on bottom	100

light and often highly pigmented. It is possible that algae in contracted polyps are shielded from light and thus photosynthesis is retarded or prevented.

B. Light intensities (see Table II).

C. Boring algae

The only genus of alga observed in skeletons of living coral in this study is *Ostreobium* (Bornet and Flahault, 1889), a siphonaceous green alga. In massive corals such as the dominant Caribbean reef-builder *Montastrea annularis* there are alternating green and white layers arranged concentrically around the point of first growth. These layers are seen as dark bands in the split coral head shown in Figure 2. Each green band is formed at the growing surface of the coral and although the algal filaments are inside the aragonite of the skeleton, they extend to the very surface of the skeleton and thus lie within 0.1 mm. of the skeletogenic tissues of the coral. It is possible that they contact coral tissue. Algal filaments can be isolated from any of the green bands in a coral head. In healthy living heads with few associated boring organisms, only *Ostreobium* was found.

However, in the microscopic examination of fixed material, the green bands found within 1 cm. of the surface of the colony were the only green bands containing algal filaments with recognizable chloroplasts and whose entire cytoplast had the appearance of one which had been fixed while alive. Only the very outside green band had a preponderance of algal filaments that appeared, by such criteria, to be living. In deeper green bands a larger proportion of the green pigment was seen to be in the mineral of the skeleton. It presumably had diffused out of the filaments and had been adsorbed by the aragonite of the skeleton.

In one of the specimens of *D. stokesii*, whose skeleton and included algae were examined by respirometry, counts of algal filaments were made per unit volume of solid skeleton. Since filaments of *Ostreobium* have no cross-walls, cell counts could not be made. However, in solid skeleton where there is relative homogeneity of

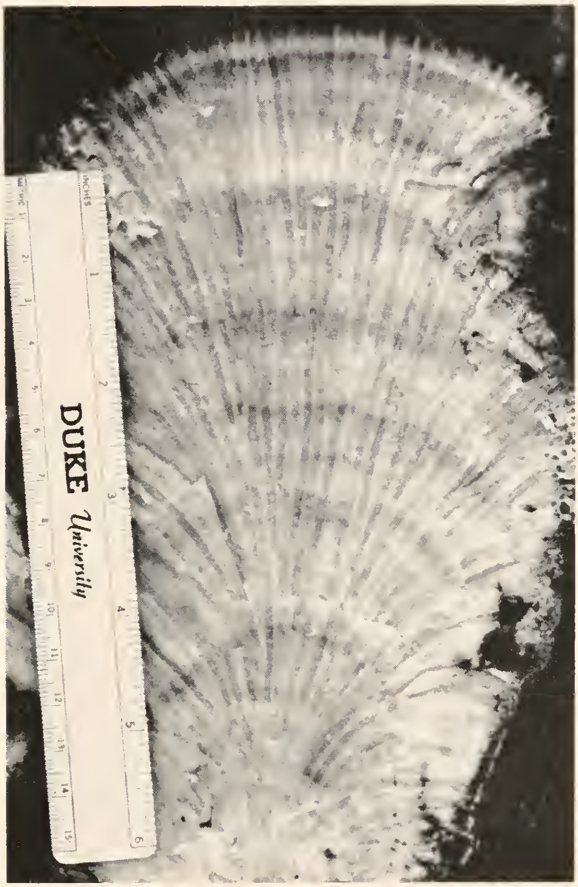

FIGURE 2. Internal (broken) surface of a head of the stony coral, *Montastrea annularis,* that has been split vertically. Concentric dark bands are the green bands referred to in the text. Vertical lines are now empty calices of individual polyps. The living coral tissue occupies the topmost layer of ca. 3 mm. thickness.

structure over volumes as large a few mm.^{3}, the algal filaments grow more or less straight and parallel to each other. In any particular field of view, the number of forks is roughly equal to the number of filament terminations. Filaments are assumed to be straight and perfectly cylindrical in the calculations. Since neither is precisely the case, the estimate of volume of algal filaments is lower than the actual value. By count in volumes of the order of 0.01 mm.^{3} in the darkest green zone, there are approximately 1500 algal filaments per mm.^{3}. At an average diameter of 10 μ per filament, the skeleton would contain 12% by volume of algae.

D. Pigments

The layer of coral polyps with its contained zooxanthellae forms a continuous sheet over the surface of the colony. The photosynthetic pigments of these algae

TABLE III

Light penetration into whole heads and clean skeleton of Montastrea annularis

Specimen	Depth of sensor in head in mm. from lighted surface	Light intensity at surface of coral in footcandles	% Light transmitted
Head #1	16	6600	0.20
Head #2	10	4500	0.78
Head #3	10	5200	0.15
Head #4	5	6500	0.92
Head #5	4	3000	1.00
Tangential slice from top of head	5 mm. thick	1450	1.9
Tangential slice of clean skeleton	10 mm. thick	3000	3.0
Radial slice of clean skeleton	10 mm. thick	3500	2.8

color the coral olive green to brown. A species such as *Montastrea annularis* may be a dark chocolate brown in color. The same species will appear nearly colorless without the zooxanthellae. The underlying chlorophyll of the boring green filaments rarely shows by inspection from the outside. Some corals, such as species of *Porites*, have smaller amounts of brown animal pigments.

A square column extending from the surface down into the coral head was cut with a glass-cutting saw. The successive colored layers were then sliced off, ground and extracted, and absorption spectra obtained with a spectrophotometer. The 2-mm. surface layer contained all the coral polyps and thus all the zooxanthellae plus a small amount of boring green algae. All succeeding layers below had only green filaments.

The absorption curve of the surface layer showed mostly accessory pigment (carotenoids) bands with the chlorophyll only scarcely visible. We estimate that a minimum of 80% of incident light is absorbed by accessory pigments. It seems unreasonable to give the zooxanthellae a less important role in the total oxygen exchange of a coral head than the subsurface green filaments only because they have less extractable chlorophyll (Odum and Odum, 1955).

Extracts of the green bands in the skeleton below the polyp layer (Fig. 2) showed only chlorophyll-type absorption peaks. Chlorophyll and its degradation product phaeophytin were determined after the method of Yentsch and Menzel (1963). A large part of the green coloring—particularly below the topmost band—was due to phaeophytin, the magnesium-free form of chlorophyll. This fraction cannot be active photosynthetically. Three things (1) the fact that only boring algae of the outermost layer appear to be intact, (2) the photosynthetically inactive nature of the subsurface layers combined with the very low light levels beneath the outer layer, and (3) our inability to measure photosynthesis when the coral tissue was removed, make it difficult for us to conclude that boring algae are major producers in coral heads from Florida reefs.

DISCUSSION OF EXPERIMENTS

The respirometric data here concern coral colonies in confined spaces for short-term experiments. Thus discussion preceding conclusions will be related to this situation only. The work was done out of interest in the ecology of corals and their associated algae. We will put forth our own suggestions and hypotheses on these matters following conclusions drawn from the data.

Some approximations are necessary for the calculations whose results appear in Table I. The respiratory consumption of oxygen in some species is markedly dependent on oxygen tension. We chose the value at air saturation since this is the range where the coral must operate in nature. The estimate of area for some of the branching species may be as much as 25% in error.

Several facts are clear from the oxygen-time curves such as Figure 1 and the resulting data in Table I. All coral species were able to photosynthesize more than the respiratory needs of the entire head. In all but one, *Mussa*, this gross productivity was more than twice as great, so that 12 hours of usable light would more than make up for the 24-hour respiratory need. Compensation intensity was usually 300–500 footcandles, much less than the average illumination level (Table II). Thus most shallow-water corals should be photosynthesizing more than all their respiratory needs.

Any artifact of handling or making a measurement in a closed chamber should reduce the rate of photosynthesis. Thus the productivity measurements given here are a minimum. In spite of this, the lowest productivity we record from a coral head is higher than that for any planktonic situation in the open sea. The highest, at 10 g. C/m.^{2}/day, is within a factor of 2 of Ryther's (1959) estimate of the theoretical maximum based on quantum efficiency and available sunlight. Such high values of productivity indicate that most of the light incident on a coral is absorbed by photosynthetic pigment. At illumination levels around compensation the computed photosynthetic efficiencies were about 10%, which is also close to the theoretical maximum.

The light reaching the boring filamentous algae in the coral skeleton is low, about 1–2% of that incident on the outer coral surface. The latter in clear weather may be 2500 to 5000 footcandles. Thus the upper filamentous green layer would have only 25 to 50 footcandles available. If it used all of this with the expected quantum efficiency the resulting carbon fixation could only be 0.2 to 0.4 g. C/m.^{2}/day, a small part of the observed for whole coral heads. It is irrelevant to consider whether the algae have a low compensation intensity since the net production can never be larger than this. From this and the fact that gorgonian corals contain no filamentous algae and still show high photosynthetic rates, we conclude that skeleton-boring algae are responsible for less than 10% of the total primary productivity of a coral head.

Except for Beyers (1966) and Roffman (1966), all previous workers have reported gas exchange in corals per unit nitrogen or chlorophyll or weight of coral. Since these parameters vary significantly among species and even within a single coral head, results based on them are not comparable and are difficult to interpret at all. We believe the only basis for comparison is the projected surface area of the colony that receives light. Since sunlight shows little directionality in the habitats of the corals we chose for experimentation, total surface area of living tissue was estimated.

General Discussion

We are concerned here with how much photosynthesis occurs on a coral reef in nature, which of the associated plants is responsible for this, and how it potentially may influence the coral. By primary productivity we mean the rate of carbon fixed per area of the reef. We have used the usual units of grams of carbon × $meter^{-2}$ × day^{-1}. In any square meter of reef surface there can be many square meters of coral surface. In an area of crowded branching corals, the potentially high productivity is not realized because of mutual shading. The analogous case of a dense sea weed stand has recently been worked out by Kanwisher (1966).

When oxygen production was measured at several light levels, the resulting curve showed a linear increase with intensity up to saturation (usually about 2000 footcandles) and then little further increase beyond this. As in sea weeds (Kanwisher, 1966) there was no sign of photosynthetic inhibition at values approaching full sunshine.

Full sunshine gives a theoretical maximum of about 100 g. C/$m.^2$/day. The intensity at which saturation occurs is about $\frac{1}{5}$ of sunshine. This immediately reduces the production to 20 g. C/$m.^2$/day since all excess quanta are wasted. As shown with sea weeds the potential photosynthesis per unit area may be many times this. But it cannot be realized because there is not a dense enough flux of light any place in nature. Thus it is ecologically invalid to relate photosynthesis to the amount of pigment, the weight of tissue, etc., since the mere capacity for photosynthesis does not guarantee that it will be used. In spite of Beyers' findings (1963, 1966) and warning (1965, p. 73) concerning the variation in photosynthetic rates of organisms over normal days, our experiments have all been of short duration. Roffman (personal communication) finds the same decrease in photosynthetic rate in both Caribbean and Pacific corals in short-term experiments. We offer no explanation for this phenomenon nor for our failure to observe it.

The filamentous green algae seem unlikely as a major source of photosynthetic gas exchange because of the long diffusion path to the outside. Both time and rate play a part in our formulation of such a conjecture. When the light intensity on a coral was changed, the oxygen curve reached its new slope in 3 to 5 minutes. This includes time for mixing in the chamber and the response time of the electrode, as well as the establishment of the new diffusion gradient within the coral. From the known diffusivity of dissolved oxygen, it follows that the distance from the major source of photosynthesis to the sea water outside is less than 1 mm. If we consider a layer 1 cm. below the surface, it would take 12 hours to reach 95% of its equilibrium gradient. In addition, the resulting flux of oxygen outward would be less than 1% of what we measured, even with a pO_2 of 1 atmosphere in the algal layer. If the deeper green layers were contributing importantly in the oxygen flux, it would be necessary to use something analogous to the extensive capillary vascular system of higher animals where mass transfer rather than diffusion is the dominant process. The volume of water in the algal channels represents too small a reservoir of nutrients and CO_2 for more than a few minutes of rapid photosynthesis. For these reasons we feel that the underlying green algae are too isolated to be contributing a major component of the gas exchange we are observing. There is always danger in extrapolating from results of experiments on Caribbean corals to the situation in the tropical Pacific Ocean. Since no worker has published comparisons of corals or their included algae from the two regions, one cannot estimate the error involved in extrapolations from our results to Pacific corals. Since our experimental corals were taken from depths of 5 to 9 m., they are perhaps still less comparable to the Pacific corals that have given rise to speculation on these matters. Total pigmentation and even morphology of coral polyps varies considerably from the top of a large massive head of *Porites,* for example, that may be just sublittoral, to the sides and bottom of the same head 2 m. further from the sea surface. It is possible that in very shallow water the pigmentation of corals and their algal components may be balanced in such a way that the latter are the chief primary producers. We would like to see both physiological data and pigment analyses to test this hypothesis.

Although the green algae appear to contribute at best only a minor part to coral productivity, it is interesting in terms of their own physiology that they are able to grow at all under such conditions of limited light and diffusion access to the outside. Since we are unable to detect any photosynthetic oxygen production by them, we cannot say what their compensating light intensity is. Sea weeds may have a value as low as 25 footcandles (Kanwisher, 1966). This is approximately the level of light penetrating the overlying polyp layer. Thus it is not impossible that the uppermost green layer can photosynthetically produce its respiratory needs and perhaps grow slowly. We would also like to point out the possibility of heterotrophic nutrition which is common among diatoms (Lewin, 1953). One of us (JWK) has frequently found filamentous green algae in dark anaerobic sediments where heterotrophic growth would seem the only explanation.

Oxygen measurements such as ours tell nothing about the eventual fate of the fixed carbon, but the possible alternatives can be inspected. First, the production may be used in the growth of the zooxanthellae. The excess cells produced, if any, can either be released to the outside or consumed by the coral itself. In either case there could be a rapid growth with a doubling of zooxanthellae numbers at least once a day (Kanwisher, 1966), and such rates are characteristic of single-celled plants. Only once in 30 hours' SCUBA diving, day and night, did we observe a coral, *Diploria strigosa,* extruding strings of brown slime that may have contained zooxanthellae. Goreau and Goreau (1960) and Muscatine (1967) have provided information that supports the notion that photosynthates are released by the zooxanthellae and are taken up by animal cells.

In summary, it seems to us that trophic interrelationships of corals and their included algae, both intracellular and skeletal-boring, are not yet fully understood. Experimental information thus far indicates that zooxanthellae can produce more than enough fixed carbon compounds to compensate for the carbon lost through respiration of the coral head. The excess, if it is real, must represent a net export from the coral reef, probably either as dissolved organic material or as detritus. One can be sure that the nutritional story of boring algae will be an interesting one, but we feel that the role they play in the primary productivity of the entire coral head will be found to be a minor one.

SUMMARY

Oxygen exchange is reported for 14 species of Florida reef corals in the dark and at different light intensities. Oxygen tension was monitored with a recording polarographic electrode. Results are given as grams of carbon fixed per square meter of coral surface per day, and compensation light intensities were given for each species. Maximum ratios of photosynthesis to respiration varied from 1.9 to 5.8. Boring, filamentous green algae living in the skeleton of *Dichocoenia stokesii* were not observed to change their rate of oxygen consumption from dark conditions up to 5000 footcandles of light. It is concluded that reef corals are among the most productive organisms known and that, in Florida corals, the boring green algae contribute very little indeed to this productivity. Some data are given on the light impinging on Florida reefs and the light penetrating coral skeleton. Reasons for believing boring green algae are of minor importance are given in the discussion.

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UPTAKE AND ASSIMILATION OF AMINO ACIDS BY PLATYMONAS [1]

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Many algae, including some marine phytoplankters, behave heterotrophically in laboratory culture. Organic compounds have been systematically employed in algal culture since 1905 (Treboux). Glucose, pyruvate, and acetate commonly support or augment growth. Amino acids may serve as acceptable nitrogen sources in culture (Algeus, 1948; Arnow *et al.,* 1953; Ghosh and Burris, 1950; Pintner and Provasoli, 1963).

Such compounds are typically supplied in culture media at concentrations which are several orders of magnitude higher than concentrations which are possible in the normal environment. Studies of organic micronutrients provide a clear exception to this statement. Culture of algae can be an extremely sensitive technique for determination of vitamin B_{12} and other compounds in natural waters (Hutner and Provasoli, 1964). However there has not been comparable laboratory investigation of the possibility that part of the requirement for carbon and nitrogen may be met heterotrophically under normal conditions.

Heterotrophy has been discussed repeatedly as a possible factor in algal blooms (*e. g.,* Provasoli, 1960). Also, recent evidence has been presented for the existence of marine phytoplankters in the aphotic zone which may survive heterotrophically. Kimball *et al.* (1963) collected phytoplankton in oceanic aphotic zones, and confirmed the presence of chlorophyll *via* red fluorescence. Large numbers of viable unicellular flagellates have been collected throughout the aphotic regions of the north Atlantic (Fournier, 1966). Further, diatoms obtained in abyssal mud samples contained protoplasm and could be cultured at high pressures. Wood (1956) strongly supports the conclusion that these diatoms are autochthonous.

An ecologically meaningful investigation of uptake and assimilation of organic compounds must take account of very low naturally occurring concentrations. Amino acids were selected partly because recent information is available concerning levels in marine and estuarine waters. Langley Wood (1965) has reported free amino acid concentrations of 1 to 3 μmoles/liter. Similar values have been published by other authors (Chau and Riley, 1966; Degens *et al.,* 1964). Also, accumulation of amino acids has been reported in a wide variety of soft-bodied invertebrates (Stephens, 1963, 1964, 1967). Comparisons with algae may be informative. Finally, nitrogen is often an apparent limiting factor in algal populations. Naturally occurring free amino acids may contribute to the nitrogen needs of these organisms.

Platymonas was chosen as an experimental organism after preliminary observations demonstrated that it accumulated glycine very rapidly from dilute solution.

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It is occasionally abundant in phytoplankton (Margaleff, 1946) and is also found in high concentration in tidepools (Lewis and Taylor, 1921).

MATERIALS AND METHODS

A culture of *Platymonas* was obtained from Dr. H. A. Lowenstam at the California Institute of Technology in the fall of 1965. The organism has been identified as *P. subcordiformis* (Hazen, 1921) on the basis of taxonomic characters listed by Kylin (Margaleff, 1946). Constant-volume 1-liter cultures were maintained at 28° C. in a medium containing 2×10^{-3} M KNO_3, 2×10^{-4} M K_2HPO_4, 10^{-5} M $FeCl_3$, and 0.001% EDTA in artificial sea water (Instant Ocean, Aquarium Systems Inc., Ohio). Sixteen hours of light per day were provided by cool white fluorescent fixtures. Cultures were vigorously aerated with 5% CO_2 in air. The pH varied from 6.0 to 7.5. Cells were also successfully grown on 2% agar made up in the culture medium. Liquid cultures were inoculated from sterile agar colonies to minimize bacterial contamination. Cell numbers in liquid culture were routinely determined colorimetrically. A standard curve was prepared relating optical density at 4000 Å to hemacytometer counts of formalin-fixed cells.

For measurement of uptake at low substrate concentration, amino acids uniformly labeled with C^{14} were added to a suspension of *Platymonas*. Cells were harvested in the log phase of growth, washed, and resuspended in filtered artificial sea water. Substrate concentrations greater than 10^{-6} molar were obtained using C^{12} amino acids to which tracer amounts of C^{14}-labeled material were added. Suspensions were incubated on a shaker bath at 28° C. One-ml. samples were collected on Millipore membranes (HAWP, 0.45 μ) at timed intervals and washed with 10 ml. artificial sea water. The dry filters were glued to aluminum planchets, and the radioactivity was measured using a thin-window gas flow detector. The radioactivity in the medium retained by the filter was about 1% of the activity found in the cells after an hour.

The disappearance of activity from the medium was also measured. Samples of the filtrate were dried on aluminum planchets. It was found that dilution of these filtrate samples with 19 volumes of distilled water gave an effective sample thickness equal to that of the cells retained on the filters. Consequently this procedure was adopted to facilitate comparison of radioactivity levels in the cells and in the medium (Fig. 1).

RESULTS

Amino acid uptake

Growth curves for cultures supplied with three nitrogen sources are presented in Figure 2. Equivalent amounts of nitrogen provided as glycine or as potassium nitrate supported growth equally well. The doubling time in the log plase was approximately 22 hours. It is apparent that the same weight of nitrogen supplied as ammonium phosphate supported growth only after a longer lag period.

A number of amino acids were accumulated by *Platymonas* from very dilute solution. Table I lists the percentage of radioactivity removed from the medium by 2×10^6 cells/ml. after 30 minutes. Amino acids representing major chemical groups (basic, acidic, aliphatic, aromatic) were accumulated at comparable rates.

Only negligible amounts of glucose got into the cells. Uptake rates were directly proportional to cell numbers (Table II) in the range tested. Five × 10^5 cells/ml. gave linear uptake for about 30 minutes and was selected as a standard cell concentration for further studies.

Bacteria were occasionally visible with the microscope in older cultures, but control experiments, utilizing heavy doses of antibiotics (neomycin, streptomycin, penicillin G at 100 mg./l.), and repeated washing of cells produced no decrease in uptake. In addition, autoradiography of cell suspensions exposed for a brief period to H^3-glycine demonstrated that the radioactivity was associated with *Platymonas* cells.

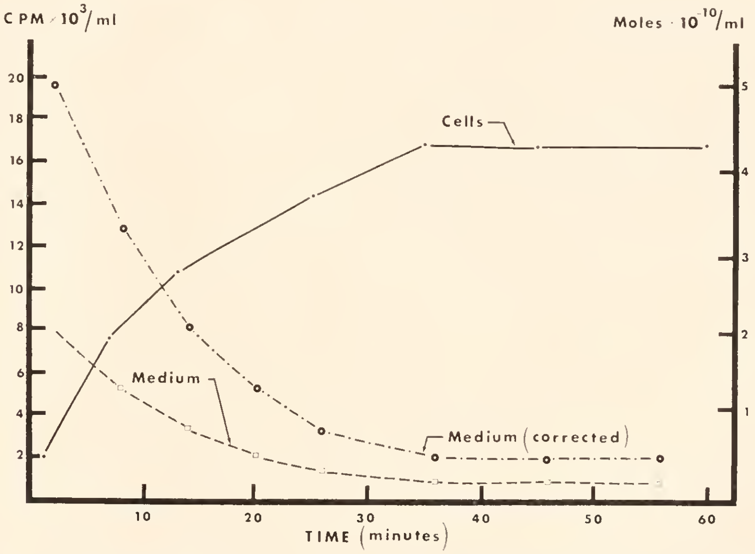

FIGURE 1. Uptake of radioactive glycine by *Platymonas* and concomitant disappearance of radioactivity from the medium. Cell concentration = 4 × 10^5 cells/ml., glycine = 5 × 10^{-7} *M.* [⊙] CPM/ml. cells. [□] CPM/ml. medium. [○] CPM/ml. medium diluted with distilled water to reduce apparent sample thickness (see text). CPM in cell samples [⊙] and corrected medium samples [○] are converted to moles glycine (right ordinate).

Amino acid uptake at high concentration was demonstrated by following the concentration in the ambient medium colorimetrically. The ninhydrin technique used was that of Clark (1964) as described by Stephens and Virkar (1966). When glycine was supplied at 10^{-4} moles/liter in a suspension of 2 × 10^6 cells per ml., 67% was removed in one hour. After cells had accumulated labeled amino acid, incubation in fresh medium for 4 hours did not leach out activity significantly above background.

Figure 3 illustrates typical uptake curves at four concentrations of glycine. Slopes (uptake velocity) were determined using the least squares method for 13 such curves at ambient concentrations ranging from 10^{-4} to 1.4 × 10^{-6} molar glycine. A plot of the reciprocal of uptake velocity against the reciprocal of ambient con-

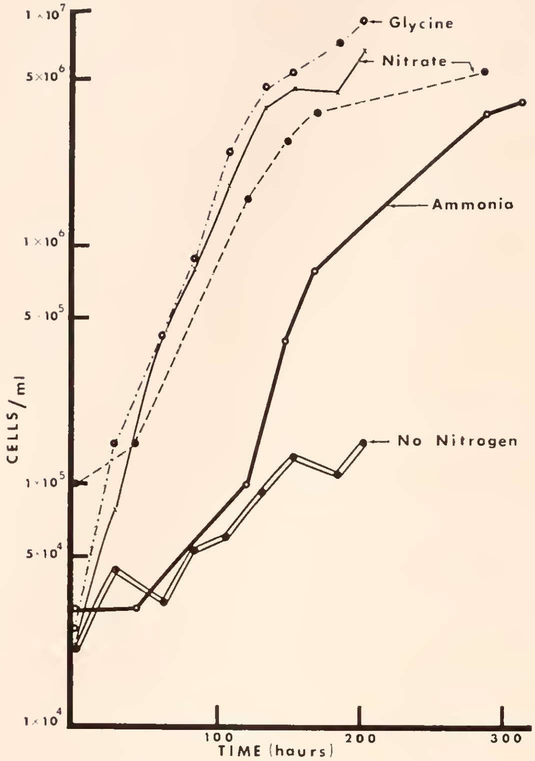

FIGURE 2. *Platymonas* growth curves. Equivalent weights of nitrogen (30 mg./l.) as glycine, K NO_3, and $(NH_4)_2$ HPO_4 were supplied. Cell number was determined colorimetrically.

centration is presented as Figure 4. This is equivalent to a Lineweaver-Burk plot and permits estimation of maximum velocity from the intercept of the line. The apparent maximum velocity is 1.3×10^{-10} moles of glycine/minute/5×10^5 cells. The straight line suggests that the rate of uptake is limited by an absorptive step at high concentrations. The process of accumulation of radioactivity in the form

TABLE I

Uptake of amino acids by suspensions of 2×10^6 cells per ml. Per cent of substrate accumulated after 30 minutes is shown at substrate concentrations ranging from 1×10^{-6} to 2×10^{-7} molar

	% Accumulation
Arginine	>95
Glutamic acid	65
Glycine	>95
Phenylalanine	50
Valine	90
Glucose	4

Table II

Uptake rates of glycine at three cell concentrations. Cells were exposed to 7×10^{-7} M glycine-C^{14} for 30 minutes

Cell concentration (number per ml.)	Ratio	Uptake rate (cpm/min.)	Ratio
3×10^5	1	90	1
1×10^6	3.3	270	3.0
2.6×10^6	8.7	775	8.6

of glycine-C^{14} continued at concentrations as low as 5×10^{-8} molar; a suspension of 10^6 cells per ml. removed 96% of the radioactivity after 60 minutes.

Assimilation

Cells incubated with glycine-C^{14} were collected at intervals and extracted with 80% ethanol. The extract was evaporated to dryness and the residue dissolved in 10% isopropanol. Ten to 20 microliters of this solution were spotted on Whatman #1 filter paper. An ascending separation was carried out in 2 dimensions with the solvent systems: butanol, acetic acid, water, 12:3:5, v/v/v; phenol, water 18:5, w/v. Autoradiograms (Kodak "No-Screen" medical x-ray film) of resulting

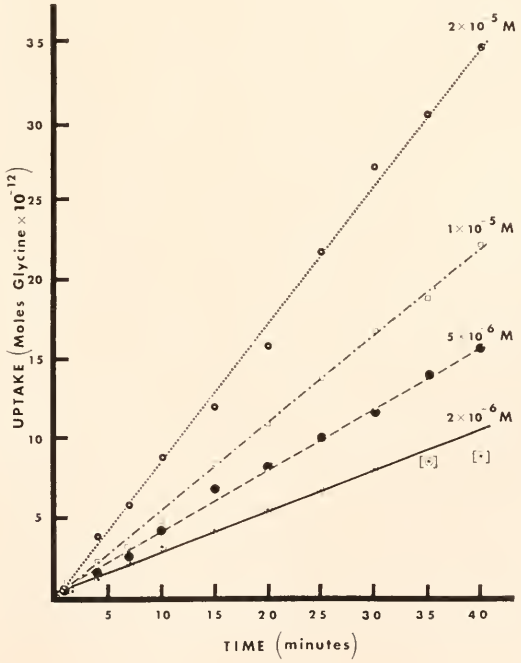

Figure 3. Uptake of glycine-C^{14} at 4 concentrations. Points are 1-ml. samples containing 5×10^5 cells. Lines obtained with the least squares method. [⊙] indicates decrease in uptake rate due to depletion of glycine in the medium.

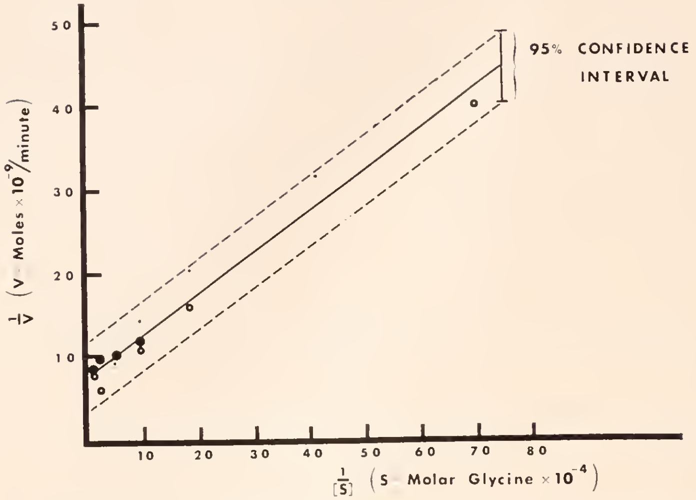

FIGURE 4. Double reciprocal plot relating uptake velocity to ambient glycine concentration. Each point represents a rate determined from curves like those in Figure 3. ⊙, ●, ○, are from separate cultures. The line was fitted by the least squares method.

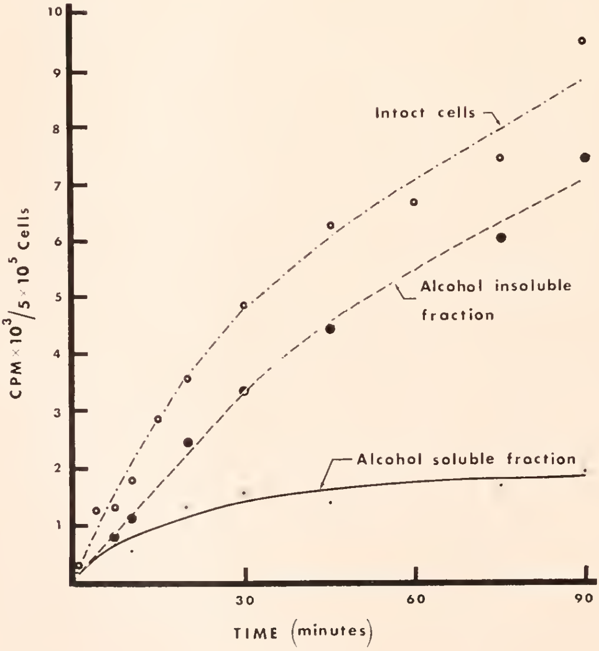

FIGURE 5. Distribution of radioactive glycine (4×10^{-7} M). [○] Total radioactivity in intact cells. [⊙] CPM in alcohol extract. [●] Radioactivity remaining in cell residue (alcohol-insoluble fraction).

chromatograms were exposed for one week. The chromatogram was sprayed with ninhydrin reagent to identify amino acids. After 5 minutes of incubation, most of the alcohol-soluble radioactivity in the cells was present as glycine (identified by co-chromatography of an authentic sample). After 60 minutes incubation, most of the radioactivity appeared as three different spots although glycine-C^{14} was still present. The spots were not identified.

Radioactivity in cells incubated with glycine or phenylalanine was separated into alcohol-soluble and alcohol-insoluble fractions. Radioactive cells were collected on Gelman (Acropore, alcohol-insoluble) filters. Activity was measured in intact cells before extraction with 10 ml. of 80% ethanol for 10 minutes. Radioactivity in 0.5-ml. aliquots of the alcohol was determined. Finally, radioactivity remaining on filters (the alcohol-insoluble fraction) was measured. Figure 5 presents a typical set of observations. The alcohol-soluble fraction of the radioactivity accumulated by the cells rapidly reached a plateau. Continued uptake from the medium appeared to depend on assimilation into other compounds, presumably polypeptides or proteins.

Evolution of $C^{14}O_2$ was observed in cells suspended in 10^{-6} *M* glycine-C^{14}. $C^{14}O_2$ was identified qualitatively by a Conway diffusion technique. CO_2 was trapped in 10% KOH. Small amounts of labeled CO_2 could be found after 2 hours of incubation, indicating that the accumulated glycine was entering oxidative pathways.

Table III

Nitrogen content of cells as a function of age of culture

Culture	mg. N per 10^8 cells $n = 3$	Nitrogen % dry wt	Culture age
1	1.33 ± .02	7.1	log phase
2	1.15 ± .13	7.2	end of log
3	0.73 ± .05	4.7	past log phase

Total cell nitrogen was determined with the semi-micro Kjeldahl technique described by Steyermark (1961). Dry weight of cells was found by collecting 10^7 cells on preweighed filters, washing quickly with distilled water, and drying to constant weight at 70° C. The resulting values (Table III) are consistent with published data for other green algae. The lowest value was found in the oldest culture.

Discussion

Platymonas subcordiformis is capable of rapid accumulation and assimilation of all amino acids tested from very dilute solutions. Glycine and nitrate are equally effective nitrogen sources when supplied at high concentration (2×10^{-3} *M*). It is of interest to consider whether a significant fraction of the nitrogen requirement of the cells might be provided at ambient concentrations which obtain in nature.

At 1.0 micromole of glycine per liter, approximately 34×10^{-10} moles of glycine per minute per 10^8 cells can be accumulated. This uptake rate is derived from

Figure 4. The nitrogen taken up during the observed doubling time of 22 hours can be obtained from the following relation (*cf.* Fencl, 1966) where

U = uptake in moles

N = cell number

r = specific growth rate
(ln 2/doubling time)

k = uptake rate

t = time

$$\frac{dU}{dt} = kN_0e^{rt}$$

$$U = \frac{kN_0}{{}_t\, r}\left[e^{rt_1} - e^{rt_0}\right]$$

For t_1 equal to the doubling time and t_0 equal to 0, this equation simplifies to $U = kN_0/r\ [2 - 1]$ or $U = kN_0/r$. Substituting measured values for r and k and taking N_0 as 10^8 cells, we obtain 0.091 mg. nitrogen. This is 7% to 13% of the nitrogen assimilated by the cells in culture (Table III). Hence, uptake from a glycine solution at 10^{-6} moles/liter provides for roughly 10% of the nitrogen requirement of the cells. This represents a minimum estimate of the probable contribution of free amino acids under natural circumstances. The estimate is based on cells in the log phase of growth with excess nitrate and optimum light conditions. The doubling time is probably longer in nature under usual circumstances. In addition these conditions maximize the nitrogen content of the cells. Fowden (1962) stated that nitrogen content in starved algae may decrease from 8% to less than 1%. Thus the probable contribution of free amino acids in the environment will be greater than was calculated above. Most or all of the required nitrogen might well be provided by this mechanism under selected natural conditions.

Algeus (1948) noted that some fresh-water algae could utilize amino acids as a nitrogen source. He determined the pH of cultures as they aged and concluded that the algae were deaminating the amino acids and the resulting ammonia was being used as a source of nitrogen. A mechanism of this kind is very unlikely in *Platymonas.* Chromatographic evidence indicated that glycine does enter the cells. Repeated efforts to demonstrate ammonia in cultures incubated with glycine concentrations of 10^{-4} moles/liter for a period of 24 hours were negative. Also ammonium phosphate supplied to these cells was utilized only after a considerable lag period (Fig. 1) although a possible inhibiting effect of ammonium at high concentrations was not investigated.

Sloan and Strickland (1966) studied the uptake of several organic compounds by algae and found that the diatom, *Thalassiosira,* removed glutamate from solution more effectively than glucose or acetate. It is impossible to make a direct comparison of their results with ours since they discarded the uptake during the first hour as a blank. However, the rates they report are lower than those measured for *Platymonas* by at least an order of magnitude. There is little additional information available on the distribution of this capacity among phytoplankters but we are preparing a report indicating rapid uptake and assimilation in several genera of intertidal macroscopic algae.

The uptake and assimilation of amino acids by *Platymonas* differs from that reported for animals by Stephens (1967) in two respects. Soft-bodied marine invertebrates typically have large free amino acid pools and show much slower rates of uptake into the alcohol-insoluble fraction of the organism. Also no rapid conversion among compounds in the alcohol-soluble fraction such as that reported here has been detected.

It has been assumed that short-term measurements presented here are valid over extended periods. Uptake of glycine has been observed qualitatively for periods as long as 20 hours. Reliable quantitative data for periods longer than 2 to 4 hours have not been obtained.

The discussion has been presented in terms of nitrogen but a similar calculation could be adduced for organic carbon. Cell carbon was not measured but if a figure of 30% is accepted from the literature (Wimpenny, 1966), the contribution of organic carbon by uptake from 10^{-6} molar solutions of amino acid is substantial. Assuming a reasonable mixture of amino acids in natural circumstances, 10% of cell carbon is provided during log phase growth. Higher fractions can be speculatively defended under less ideal conditions as has been suggested above.

It seems more reasonable to consider this mechanism in relation to nitrogen requirements in photosynthetic cells. In any case, the uptake of amino acids is not influenced by the presence of nitrate in medium. There is no modification of the uptake rate of labeled glycine in the presence of potassium nitrate at concentrations 100 to 1000 times greater than that of the amino acid. Consequently, as pointed out by E. J. F. Wood (1965), studies which assume inorganic nitrogen to be equivalent to the total nitrogen available for plant productivity should be treated with reserve.

In conclusion, the evidence for a normal role of heterotrophy in the economy of at least some algae is now quite substantial. In particular, *Platymonas* can obtain significant amounts of nitrogen and carbon from ambient concentrations of amino acids which lie in the normal range observed in the habitat of this organism.

SUMMARY

The marine flagellate *Platymonas subcordiformis* rapidly takes up amino acids at concentrations likely to be found in nature. The relation between velocity of uptake and substrate concentration was determined, and the apparent maximum uptake velocity estimated. Accumulated C^{14} amino acids were assimilated into alcohol-insoluble compounds and entered oxidative pathways as shown by detection of evolved $C^{14}O_2$. The contribution of the uptake mechanism at an ambient glycine concentration of 1.0 μmole/l. to nitrogen requirement of the cell was found to be roughly 10% for optimal laboratory growth conditions. In nature, where growth conditions are probably less favorable, the contribution would be considerably greater. This provides further support for a normal role of heterotrophy in phytoplankters.

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ENZYMES OF THE ACCESSORY BORING ORGAN OF THE MURICID GASTROPOD UROSALPINX CINEREA FOLLYENSIS. I. AEROBIC AND RELATED OXIDATIVE SYSTEMS [1]

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In continuation of studies of the demineralization mechanism of the accessory boring organ (ABO) of the shell-penetrating snail *Urosalpinx,* by Carriker, Scott and Martin (1963), Provenza, Nylen and Carriker (1966; and unpublished observations) demonstrated by means of electron microscopy that the secretory cells of the ABO contain dense concentrations of mitochrondria. Preliminary observations in the course of these studies suggested that numbers and size of mitochondria were significantly greater in secretory cells of ABO's from actively boring snails than of ABO's from non-boring or resting snails.

These observations are of interest because (1) mitochondria are the major sites of cell respiration, and (2) the secretory function of many exocrine glands is known to be dependent upon an active aerobic oxidative metabolism. As an example, in vertebrate salivary glands it has been well established that basal secretion, as well as any increases in *in-vivo* or *in-vitro* secretory activity, are obligatorily dependent upon oxygen utilization (for summarized references see Schneider and Person, 1960; Person *et al.,* 1961).

Because of the lack of available information in the literature concerning the intermediary metabolism of the ABO, a preliminary correlated histochemical and biochemical study of aerobic and related oxidative enzymes of the *Urosalpinx* ABO was undertaken. The objective of this was to determine whether the cytologic observations of dense populations of mitochondria in the secretory cells of the ABO indicate a significant aerobic metabolism in that organ. Observations and data reported in this paper show that the ABO does indeed possess a very active aerobic metabolism as judged by its cytochrome oxidase activity. It was also demonstrated in the laboratory that live snails will not penetrate the shell of oyster prey when their environment is depleted of oxygen.

MATERIALS AND METHODS

Specimens of *Urosalpinx cinerea* (Say) were obtained locally on intertidal rock jetties in Woods Hole for preliminary studies; results reported in this study

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are based primarily on the large *Urosalpinx cinerea follyensis* Baker shipped alive *via* air mail from Wachapreague Bay, Virginia.

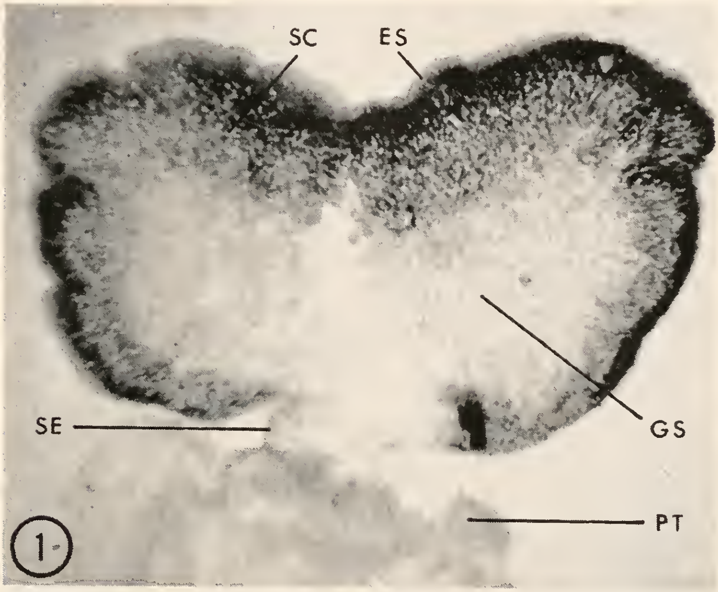
Snails were maintained in running sea water tables at the Marine Biological Laboratory, Woods Hole, where the research was conducted during the summer of 1966. For each enzyme determination, snails were removed from their shells by cracking, and the ABO's were excised cleanly under a binocular dissecting scope (see Carriker, Scott and Martin, 1963, for method). For histochemical studies, ABO's were severed at the region where the base of the ABO stalk is attached to the foot of the animal. Excised organs were immediately frozen with dry ice, and sections were cut at 8–10 μ using the cold-knife technique of Adamstone and Taylor (1948). The following histochemical procedures were performed: cytochrome oxidase (Burstone, 1960; with naphthol ASL-3G and added cytochrome *c*); succinate dehydrogenase (Nachlas *et al.*, 1957; with added menadione according to Wattenberg and Leong, 1960; and Morrison and Kronheim, 1962) and lactate dehydrogenase (Ogata and Mori, 1964). For biochemical assays, the distal secretory disk of the ABO was cut away from the stalk, leaving behind as much as possible of the non-secretory stalk tissues. Usually ABO's from 3 snails were pooled for each determination. Tissues were placed in the appropriate chilled (2°–4° C.) buffer for each assay and homogenized in a ground-glass tissue homogenizer. The following assays were performed: cytochrome oxidase (Wainio *et al.*, 1951); NADH-cytochrome *c*-reductase (Lenta and Riehl, 1960); succinate-cytochrome *c* dehydrogenase (Cooperstein *et al.*, 1950); lactate dehydrogenase (Kornberg, 1955) and glucose-6-PO_4 dehydrogenase (Noltman *et al.*, 1961). Protein determinations were by the method of Lowry *et al.* (1951).

A series of experiments were also performed in which snails were maintained in sea water, on, or in the presence of, live prey (*Crassostrea virginica*) under anaerobic conditions (N_2 atmosphere) for definite time intervals prior to biochemical and histochemical study of their ABO's. Details will be given in the appropriate section of the text.

Results

1. *Histochemical observations*

a. *Cytochrome oxidase.* The range of inhibition by cyanide was from 10^{-4} *M*, for complete, to 10^{-6} *M* for partial inhibition. Complete inhibition was given by 10^{-2} *M* azide, while inhibition by 10^{-4} *M* azide was only partial. Sections kept at 100° C. for 5 minutes were completely inactive. Omission of cytochrome *c* from the incubation mixture retarded color formation by a factor of 2 to 3. Figure 1 shows that cytochrome oxidase activity was confined almost entirely to the secretory cells (SC) of the ABO, and that neither the internal stalk tissues (GS), the stalk epithelium (SE), nor pedal tissue (PT) showed significant activity. In Figure 2 a higher magnification is shown. It may be seen in Figures 1 and 2 that most intense activity was present at the distal ends (*i.e.*, close to external surface) of the epithelial cells, and that a layer of secreted material external to the epithelial surface of the secretory cells (ES) also exhibited oxidative dye synthesis, although of lesser intensity. This secretory material presumably contains the substances which are active in chemically attacking the $CaCO_3$ crystals of shells which are



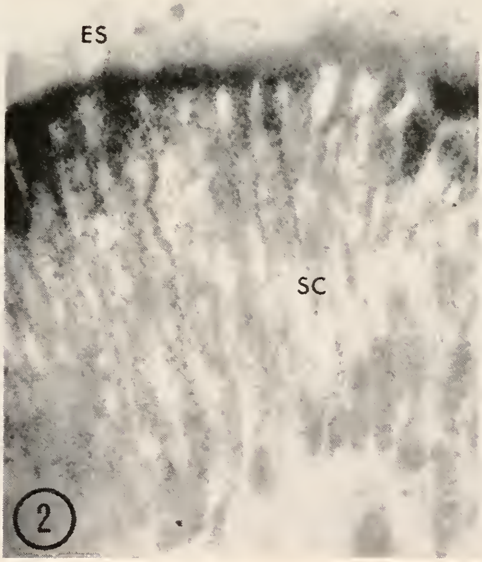


Figure 1. (Upper) Distribution of histochemical cytochrome oxidase in a sagittal freshly frozen section of *Urosalpinx* ABO. The secretory epithelial cells (SC) are intensely stained. Little or no activity is seen in the gland stroma (GS), in any of the stalk epithelium (SE) or in pedal tissue (PT). Note that externally secreted material (ES) at the outer surface of the secretory cells shows a slightly positive reaction. Magnification approximately 60 ×.

Figure 2. (Lower) Enlargement of secretory cell region from a comparable section to that seen in Figure 1. Magnification approximately 250 ×.

bored by the snails (see Carriker, Scott and Martin, 1963; and Carriker and Van Zandt, 1964). In view of the fact that cytochrome oxidase is almost always intracellular in localization, it is of considerable interest that extracellular oxidase activity should be associated with such secretions.

It should be mentioned that the rate of dye synthesis appeared to be more rapid than that customarily seen even with most actively aerobic mammalian tissues studied under similar assay conditions. Thus, in the epithelial cells of the ABO, dye formation was quite visible to the unaided eye in 5 minutes, and as seen in Figures 1 and 2 was markedly advanced by 15 minutes. Comparable color intensities in mammalian heart or kidney sections usually require 30 minutes or more of incubation time. It will be seen later that the biochemical data support this impression of greater activity in the ABO epithelial tissue.

b. *Succinate dehydrogenase.* When malonate, a classical inhibitor of succinic dehydrogenase, was made equimolar to succinate in the incubation mixture (*i.e.* 10^{-2} M), it strongly inhibited activity. Omission of succinate from the reaction mixture resulted in complete absence of color formation in the interval studied (*i.e.,* 3–10 minutes). Figure 3 shows that the distribution of succinate dehydrogenase activity almost completely paralleled that of cytochrome oxidase, with the possible exception

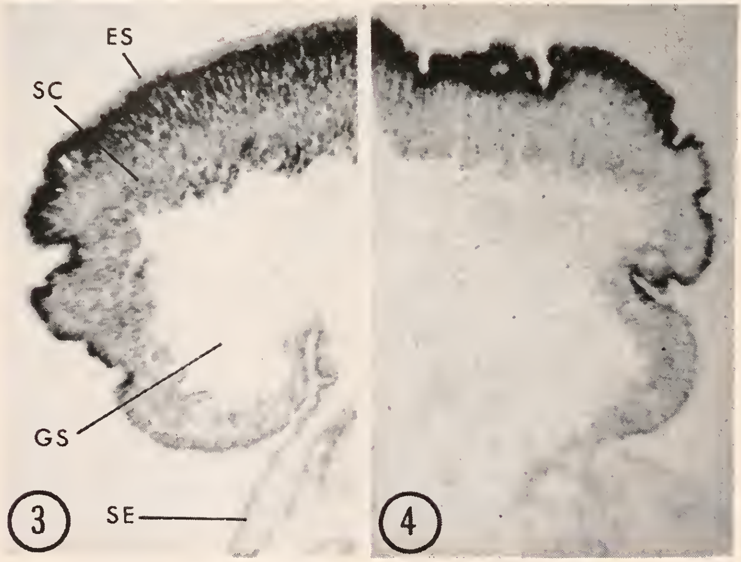


FIGURE 3. (Left) Distribution of histochemical succinate dehydrogenase in a saggital freshly frozen section of *Urosalpinx* ABO. Activity appears in locations similar to that seen for cytochrome oxidase in Figures 1 and 2; only secretory epithelial cells (SC) show activity, while gland stroma (GS) and stalk epithelium (SE) are negative. Externally secreted material (ES) at the outer surface of the secretory cells shows a positive reaction. Magnification approximately 60 ×.

FIGURE 4. (Right) Distribution of histochemical lactate dehydrogenase in a sagittal freshly frozen section of *Urosalpinx* ABO. Activity is found only in epithelial secretory cells, and primarily at the distal edge. Magnification approximately 60 ×.

that in some cells in the former system dye formation was occasionally visible in nuclei (not shown in illustrations). As was the case with cytochrome oxidase, activity was widespread throughout secretory cells, and was not seen in stalk components. Again, such activity was especially concentrated at the proximal and distal ends of cells, with the greatest concentration at the latter. Very intense dye formation occurred in 3–5 minutes.

c. *Lactate dehydrogenase.* Figure 4 shows that positive reactions for this enzyme also occurred, with widespread distribution throughout secretory cells and little if any activity in stalk components. As was the case with cytochrome oxidase and succinate dehydrogenase activities, color formation was greatest at the epithelial surface end of the cells; but unlike the former two enzymes, activity at the proximal (stalk) end of the cells was much reduced.

2. *Biochemical assays*

a. Cytochrome oxidase

1. *Preliminary observations.* Homogenates kept at 100° C. for 5 minutes were completely inactive. Oxidase activity was inhibited by 10^{-4} M cyanide and 10^{-4} M azide to the extent of 76% and 60%, respectively. On standing at 1°–2° C., homogenates progressively lost activity, so that after $3\frac{1}{2}$ hours, only 40% of initial activity remained. In beginning assays, snails were taken at random from the waters surrounding Woods Hole, and also from different sea tables in different laboratories (in which variable populations of other invertebrates from a variety of phyla were also maintained). On 18 different days, 18 separate oxidase assays were performed using 3 pooled ABO's per assay. The range of specific activities in this series of determinations was from 0.190 to 2.10 μM cytochrome *c* oxidized/mg. protein/minute (in 15 of the 18 assays); in 3 of the 18 assays absolutely no cytochrome oxidase activity could be measured. It was found that ABO's from the snails shipped from Wachapreague Bay (*U. c. follyensis*) were more consistently active, so that in the experiments which follow the gastropods of the Wachapreague Bay subspecies were employed. They were all maintained under closely similar conditions with reference to availability of live food, running sea water and temperature.

2. *Influence of physiologic status of snails on oxidase activity.* A series of experiments were performed to determine the influence of the snail's physiologic status upon oxidase activity. Snails in the sea-water table were classified according to whether they were "resting" (*i.e.*, no contact with oysters), "drilling" (*i.e.*, mounted on live oyster with evidence of incompletely drilled hole in shell) or "feeding" (*i.e.*, mounted on oyster, with perforation of shell and feeding on oyster). Table I shows that the oxidase activities of the three groups were quite similar, with no detectable differences outside the range of standard deviations.

In order to determine whether or not non-oxidase protein from the ABO and associated stalk tissues might be masking changes in specific activity of the homogenates, experiments were performed to minimize such contamination. To obtain sufficient tissue material, ABO's from 10 resting and 10 drilling *Urosalpinx* were pooled in two groups, and each group was separately homogenized. For each homogenate system, intact cells and debris were first sedimented in a centrifuge at 600 *g* for 20 minutes. The supernatant fluid was recovered and spun at 30,000 *g* for 30 minutes. The 30,000 *g* supernatant fluid was now completely devoid of oxidase activity and was discarded. The pellet was resuspended in buffer and again spun at 30,000 *g* for 30 minutes. The washed pellet was then resuspended and used for assay. This time the specific activities were: resting, 0.639; drilling, 1.040, showing that there were indeed differences between specific activities of resting and drilling glands which had been masked by indifferent protein in the homogenates.

3. *Influence of lack of oxygen upon boring behavior of snails and oxidase activity of their ABO's.* To determine the effect of oxygen deprivation upon boring behavior, two types of experiments were performed. In the first, all snails were maintained in running sea water for several days in a resting state prior to the experiment, *i.e.,* neither in contact with, nor near oysters. Approximately one dozen snails were then placed in each of two desiccators containing fresh live oysters in sea water. To set up an anaerobic group, one desiccator was alternately evacuated and flushed with N_2 atmosphere to remain at atmospheric pressure. The aerobic group was maintained in air at atmospheric pressure. In the second experiment, all snails were maintained in a drilling state prior to the experiment. They were then placed in their respective desiccators while mounted on the live oysters, and the anaerobic group was established as described above. In both experiments, following placement in the desiccators, snails were maintained aerobically or anaerobically for a period of 24 hours, following which they were removed from the oysters and their ABO's were excised for analysis. In each experiment, snails in the anaerobic desiccators were never securely attached to the oysters and were easily removed, whereas snails mounted on oyster shells under aerobic conditions were always securely attached and were removed only with considerably greater effort. It should also be noted that in the second experiment, many of the initially drilling animals, when placed under anaerobic conditions, disengaged from their underlying oyster shells and fell off. Results of oxidase determinations are given in Table II, from which it can be seen that the physiological (nutritional) status of snails prior to experiment appears to exert a significant influence upon oxidase activity of ABO's. Thus, when animals were initially resting prior to experiment (Table II, experiment 1), ABO homogenates from all aerobically maintained animals were twice as active as ABO homogenates from anaerobically maintained animals. However, when animals had been drilling prior

TABLE I

Cytochrome oxidase activity of homogenates of the accessory boring organ of Urosalpinx with reference to physiologic status of animals. (Specific activity = μ moles cytochrome c oxidized/mg. protein/minute)

Status of snails	No. of experiments	Snails per experiment	Mean specific activity	Std. deviation
Resting	8	3	0.528	0.119
Drilling	8	3	0.732	0.165
Feeding	4	3	0.531	0.092

TABLE II

Cytochrome oxidase activities of homogenates of the accessory boring organ of Urosalpinx following maintenance under anaerobic and aerobic conditions. (Specific activity = μ Moles cytochrome c oxidized/mg. protein/minute)

Status	No. of experiments	Snails per experiment	Mean specific activity	Std. deviation
Experiment 1. Snails initially resting				
Anaerobic	2	3	0.592	0.058
Aerobic	2	3	1.368	0.012
Experiment 2. Snails initially drilling				
Anaerobic	2	3	0.640	0.036
Aerobic	2	3	0.435	0.090

to the experiment, there was no significant difference in oxidase activity of ABO's from aerobic or anaerobic groups (Table II, experiment 2).

b. Other oxidative enzymes

On several occasions attempts to detect succinate-cytochrome *c* dehydrogenase, lactate dehydrogenase and glucose-6-PO_4 dehydrogenase activities in ABO homogenates by the assay procedures listed under MATERIALS AND METHODS were consistently negative. Results with NADH-cytochrome *c* reductase were extremely variable, and will therefore not be described in this paper.

DISCUSSION

The histochemical and biochemical data and observations presented above indicate the existence of a very active aerobic metabolism in the secretory cells of the *Urosalpinx* ABO which is consonant with the extended and active use of the gland in boring through the shell of bivalve prey (Carriker, Scott and Martin, 1963; Carriker and Martin, 1965). The bipolar concentration of oxidase activity (at the proximal and distal ends of the cells) suggests that transport and secretory processes at these respective sites may be supported by the aerobic systems. The similar histochemical distribution of succinate dehydrogenase and cytochrome oxidase activities is in keeping with the well known localization of both these enzymes in mitochondria. Our inability to detect succinate dehydrogenase by the biochemical assay method may have resulted from (a) the presence of endogenous inhibitors (for the biochemical assay system) in the homogenates, or (b) a requirement for a different assay system with a more suitable "poise" such as the phenazine methosulfate procedure of Singer and Kearney (1957). Similar considerations may apply in connection with the positive histochemical results for lactate dehydrogenase, and the negative results with the biochemical assay for this enzyme. These problems will be treated in a separate study in the future.

The range of specific activities of cytochrome oxidase in the biochemical assay is unusually high (0.190–2.10) for crude tissue homogenates. In two other experiments using ABO's which were frozen in dry ice immediately following excision and stored at −20° C. for 3 weeks prior to assay, homogenate specific activities were as high as 3.82. Such high cytochrome oxidase activities in whole homogenates from any animal tissues have never been reported previously to our knowledge. For comparison we have calculated specific activities for heart muscle homogenates from data in the literature obtained with similar assay methods (Dobson and Kasahara, 1964; Lang *et al.*, 1963). These authors' data convert to specific activities of 0.043–0.062 and 0.0025–0.0027, respectively, for rabbit and mouse heart muscle whole homogenates. In our own laboratory (P.P.) we obtain specific activities of 0.256–0.574 for isolated beef heart mitochondria, and values in the region of 2.30 for purer deoxycholate-solubilized and fractionated beef heart oxidase preparations (Person, Schneider and Scapa, 1961).

The lessened ability of drills to remain firmly attached to oyster shells and their inability to bore under anaerobic conditions also emphasize the aerobic nature of these phenomena and correlated well with conditions in the natural coastal habitats of the snails, where under usual conditions the water around them is highly oxygenated (Carriker, 1955).

With respect to the question asked at the initiation of this work, namely, do the electron microscopic cytologic observations of increased mitochondrial populations in secretory cells of drilling snails indicate an enhanced aerobic metabolism on the part of the gland, the present data and observations answer in the affirmative. In addition, the importance of aerobic processes in predation by the snails is also emphasized by the experiments with the live intact gastropods, in which mounting of prey and drilling behavior virtually ceased under anaerobic conditions. The exact relationship of aerobic processes within the gland to demineralization events is not yet known. It should be kept in mind, however, that shell penetration by snails involves predominant use of the ABO as compared with the radula. In this connection Carriker and Martin (1965) and Carriker and Van Zandt (1964) have shown that a typical cycle of the boring behavior of *Urosalpinx* involves apposition of the gland to shell for a period ranging from 30 to 90 minutes, during which weakening of the shell by demineralization occurs, followed by withdrawal of the gland, and rasping of the weakened shell by the radula for an interval of a few seconds to 5 minutes. Successful penetration of shelled prey involves alternate use of gland and radula in this fashion for periods ranging from a few hours for thin-shelled prey, to as long as 7 days of continuous activity for large thick-shelled prey.

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SUMMARY

1. Histochemical observations of the aerobic metabolism of the accessory boring organ (ABO) of *Urosalpinx* showed that cytochrome oxidase, succinate dehydro-

genase and lactate dehydrogenase activities are localized in the secretory cells of the distal secretory disk.

2. Correlated biochemical assays of cytochrome oxidase activity of whole homogenates of the ABO prepared from active snails gave specific activities which ranged between 0.190 to 2.10.

3. There were no differences in cytochrome oxidase specific activities using ABO whole homogenates from resting or drilling snails. However, the specific activities of mitochondrial-rich particulate fractions isolated from whole homogenates by high speed centrifugation showed that oxidase activities of drilling specimens were 1.040 as opposed to 0.639 for samples from resting specimens.

4. Snails did not bore shells of the oyster *Crassostrea virginica* when maintained in a N_2 atmosphere, and ceased boring begun prior to transfer to anaerobic conditions.

5. These data and observations were discussed in relation to physiological and ecological factors involved in predation and demineralization activities by *Urosalpinx*.

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SODIUM AND CHLORIDE BALANCE IN THE KILLIFISH FUNDULUS HETEROCLITUS

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The euryhaline killifish *Fundulus* has been used extensively in physiological research but no overall picture is available of salt balance in this fish in sea water and fresh water. Indeed knowledge of salt balance in fishes generally is still very incomplete. It has been known for half a century that marine teleosts maintain a blood concentration below that of sea water (Bottazzi, 1906). In 1930 Smith demonstrated that marine teleosts maintain water balance by drinking sea water and excreting the salt extra-renally. The availability of radioactive isotopes has provided more detailed information on salt movement in the last 15 years and has helped to show that the rate of turnover of sodium and chloride ions in several species is much greater in sea water than in fresh water (Mullins, 1950; Gordon, 1963; Motais and Maetz, 1964). Mullins attributed the high influx in sea water entirely to the drinking of the medium and Gordon (1963) and House (1963) concurred with this hypothesis, but Motais and Maetz showed that in the flounder *Platichthys*, drinking accounted for only part of the total influx. However, Motais and Maetz measured the drinking rates of only four fish and used Smith's original technique. It is of interest to note that these appear to be the first direct measurements of drinking rates since Smith's original work. In a paper which has appeared since this work was completed Motais, Romeu and Maetz (1966) have shown that the high rate of exchange in sea water teleosts is due in some fish, *e.g. Platichthys*, mainly to exchange diffusion while in others, *e.g. Fundulus*, the exchange diffusion component is small.

The present work is an attempt to provide a balance sheet for salt uptake and output in a well-known euryhaline fish. Experiments with fish are often complicated because these animals are very sensitive to handling, diuresis and other changes being induced both by mechanical damage and by shock. The present experiments have been devised to reduce handling to a minimum. To this end fish were loaded by placing them in a radioactive solution, not by injection, and drinking experiments were devised in which it was not necessary to ligate the anus.

MATERIALS AND METHODS

The fish were *Fundulus heteroclitus* weighing between 2 and 5 gm. Most of the fish were collected by the Woods Hole Marine Biological Laboratory Supply Department but in some cases, in order to keep handling to a minimum, fish were collected in glass minnow traps set for a few hours in nearby brackish ponds. The fluxes measured in these latter fish were not significantly different from those in fish from the laboratory tanks.

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Sea-water-adapted fish were kept in running sea water. Experiments were performed in sea water, an artificial fresh water of reproducible composition, and 40% sea water, approximately iso-osmotic with the blood. The sea water was pumped from some distance offshore and therefore varied slightly in salinity and temperature. The sodium content varied between 420 and 435 m*M*/L., 30.7–31.8‰ salinity, and the temperature between 17° and 20° C. except for a short time when it reached 23° C. The great majority of experiments were carried out at 20 ± 1° C. The assumed sodium and chloride contents of the sea water, used in the calculations which follow, will be 425 m*M* Na/L. and 495 m*M* Cl/L. Forty per cent sea water was prepared by dilution of sea water with tap water and "fresh water" was prepared by diluting sea water to 1 m*M*/Na/L. with distilled water in order to give a medium of known and reproducible composition. Both 40% sea water and fresh water tanks were aerated and kept in trays of running sea water so that their temperature remained within 1° C. of that of the sea water. All animals were adapted for at least three days before experiments. Fish were fed every two or three days on chopped clam.

Total body sodium and potassium were measured by dissolving weighed fish, dried with tissue paper, in a minimal quantity of concentrated nitric acid and measuring the sodium concentration on a Coleman flame photometer. Sea water sodium and potassium were also measured with the flame photometer. The sodium/potassium ratio in sea water is so high that potassium readings are enhanced, and therefore, potassium in sea water was measured using a standard containing a similar sodium/potassium ratio to sea water. Chloride was measured on a Cotlove chloridimeter, total body chloride being estimated after homogenizing fish in distilled water and centrifuging to remove the bulk of the solids.

Measurement of influxes and effluxes

The isotopes were ^{24}Na, ^{36}Cl and ^{82}Br. Chloride 36 is a weak beta-emitter and the sensitivity of the aluminum-covered scintillation crystal to ^{36}Cl is necessarily very low but it was still possible to obtain significant counts from living animals. Much greater sensitivity could have been obtained with liquid scintillation but this would have required the sacrifice of the fish. Bromide 82, a gamma-emitter, was used in a number of experiments as a substitute for chloride.

Fish were loaded by placing them in 250 ml. of aerated medium containing 50 to 250 μc. of ^{24}Na, ^{36}Cl or ^{82}Br. After a suitable period of loading the animals were washed for 5 to 10 minutes and then transferred to a 100- or 250-ml. efflux bath. At suitable intervals of time 5-ml. aliquots were removed from the efflux bath and their activity was measured using a Nuclear-Chicago crystal scintillation counter, after which the aliquots were returned to the bath. At the end of the experiment the activity remaining in the fish was determined. If A_w was the total activity in the efflux bath and A_f the activity in the fish at time T then the rate constant of the efflux, K_E, can be calculated as follows:

$$K_E = \frac{1}{T} \log_e \left(\frac{A_f + A_w}{A_f} \right) \qquad (1)$$

In sea water or 40% sea water the washing bath is effectively an infinite pool of salt but in "fresh-water" solutions the salt content of the bath was of similar magnitude to the salt content of the fish. The specific activity of the ions in the bath, therefore, changed during the course of the experiment. In these circumstances the equations for a two-compartment system were applied (Solomon, 1949). If Na_w and Na_f are the sodium contents of the medium and fish, respectively, K_1 the rate constant for the sodium in the medium, *i.e.* that fraction of sodium in the bath that exchanges per unit of time, and K_2 that for the sodium in the fish, then the flux of sodium through the fish, when in sodium balance is:

$$K_1Na_w = K_2Na_f$$

The declining activity in the medium after time T is given by

$$A_{wt} = (A_{w0} - A_{w_\infty})e^{-(K_1+K_2)T} + A_{w_\infty}$$

where A_{w0} is the initial activity in the medium, $A_{w\infty}$ is the activity in the medium at equilibrium and A_{wt} is the activity in the medium at time T. The increasing activity in the fish is given by

$$A_{ft} = A_{f_\infty}(1 - e^{-(K_1+K_2)T})$$

where A_{ft} is the activity in the fish at time T and $A_{f\infty}$ is the activity at equilibrium. Similarly, when the loaded fish is placed in an inactive medium

$$A_{ft} = (A_{fa} - A_{f_\infty})e^{-(K_1+K\ )T} + A_{f_\infty}$$

and

$$A_{wt} = A_{w_\infty}(1 - e^{-(K_1+K_2)T})$$

During the short wash between influx and efflux some activity is lost to the washing solution, which was allowed for by extrapolating $\log_e A_{ft}$ back to the time of the beginning of the wash.

Sodium ions in *Fundulus* are distributed amongst several components. The greater part of the sodium, *ca.* 45 m*M*/kg. fish, behaves as a single component. This is probably constituted by the sodium in the blood and extracellular fluids, together with most of the cellular sodium. This sodium exchanges rapidly with sodium in the medium when the fish are in sea water but slowly when the fish are in fresh water. A small part of the sodium in the fish, *ca.* 3–4 m*M*/kg. fish, exchanges only slowly with the environment. This sodium may be situated in the bone and possibly to some extent in cells and connective tissue. Sodium in the gut lumen will form a third component in sea-water-adapted fish. Motais and Maetz (1964) found that in the flounder the specific activity of the gut sodium was only half that of the plasma sodium for some time after transfer to active sea water. This gut sodium may account for much of the difference in total body sodium between sea-water- and fresh-water-adapted *Fundulus* (see below). The presence of these various compartments complicates the interpretation of gross influx and efflux rate constants.

The total ion content of an animal may be determined either by chemical analysis or from the specific activity of the loading solution and the total activity of the animal at equilibrium when

$$\frac{A_{f\infty}}{Na_f} = \frac{A_{w\infty}}{Na_w}$$

If the fish has been loaded for a long period so that the specific activity of all compartments reaches that of the loading solution and is then transferred to a large volume of inactive solution, the declining activity in the animal will be the sum of a number of exponentials. The rate constant of the decline will be highest initially as the blood/extracellular fluid compartment unloads but will become smaller as the slower compartments, in series with the blood, predominate. A logarithmic plot of the activity against time can only be graphically analyzed into a number of exponentials, if the time constants of the exponentials are widely different. If a multicompartmental animal is loaded for a short time only and then transferred to an inactive solution, the blood which exchanges directly with the medium will have the highest specific activity and effects of the slower compartments will be reduced. A rate constant determined in this way will approximate to that of the blood sodium alone. Similarly the determination of influx rate constants will also be affected by the presence of several compartments. If the ion behaves as if it were all in one compartment then the rate constant of the influx, K_1, may be calculated from the equation

$$K_1 = \frac{1}{T} \log_e \left(\frac{A_{f\infty}}{A_{f\infty} - A_{ft}} \right) \qquad (2)$$

However, when there is more than one compartment the blood/extracellular fluid sodium compartment will load more rapidly. If the influx is of short duration the influx rate constant K_1 calculated from equation (1) will represent the sodium influx expressed as a fraction of the total body sodium. However, in longer experiments, equation (2) will give progressively smaller values of K_1 as the inactive sodium remaining in the animal, $A_{f\infty} - A_{ft}$, is identified more and more with the slowest components.

To summarize: short-term influx experiments will give an effective rate constant for the whole animal while short-term efflux experiments especially if following a short-term load, will give initially a rate constant approximately to that of the fast blood/extracellular fluid compartment alone. This is borne out by the experiments. In sea water and 40% sea water the efflux rate constants calculated from equation (1) are greater than the influx constants calculated from equation (2). In fresh water where the gut sodium is small and where the exchange across the body surface is much slower than the exchanges within the animal, the rate constants are not significantly different.

In many experiments A_∞ was calculated from the specific activity of the loading solution and the average composition of fish adapted to that salinity. As the individual compositions were rather variable each rate constant calculated in this way was subject to some indeterminate error, but the means should not be effected significantly.

In the initial experiments activity in the fish was determined by dissolving the fish in concentrated nitric acid, diluting to a known volume and measuring the activity in a 5-ml. aliquot. However, it was found that with fish 2–5 gm. in weight the count for the whole fish, when placed head down in a test tube, always lay between 91% and 108% of the count of the fish when dissolved and made up to 5 cc. and the mean count of the whole fish was 96.5% ± 1.2 (N = 14) of that of the solution. In some later experiments the live fish were counted and the activity in the fish calculated from this relationship.

The rapid changes of efflux that follow changes of salinity were investigated by measuring activity released into an inactive solution. Fish adapted to sea water were loaded for 12 hours in 200 ml. of sea water containing 1 mC. ^{24}Na/L. After a two-minute wash in running sea water fish were transferred to 200 ml. of inactive sea water. Five-ml. samples were taken every five minutes, counted and returned to the bath. After a suitable period of time the fish were washed for two minutes in running tap water and transferred to 200 ml. of artificial "fresh water" and the procedure repeated. The fresh water was changed every hour. At the end of the experiment the activity remaining in the whole fish was counted. From the successive losses and the final activity, the initial and the rates of loss were calculated. The variation of the rate of uptake with the external concentration was measured as follows. A solution containing 10, or in some cases 30 mM Na/L. and 500 μc. ^{24}Na/L. was prepared. By dilution a series of solutions were prepared of the same specific activity but containing 0.1, 0.3, 1.0, 3.0 and 10 mM Na/L. A fish was first placed in the most dilute solution for 5 minutes, washed in running tap water for 5 minutes and the activity counted. It was then placed in the next most concentrated solution for 5 minutes and the procedure repeated. As the increment of activity gained by the fish in each solution was greater than the total activity gained in the less concentrated solutions, the activity gained in every solution could be obtained with reasonable accuracy.

For example, after 5 minutes successively in solutions containing 0.1, 0.3, 1, 3 and 10 mM Na/L., a fish counted 822, 3165, 8773, 18,604 and 36,427 cpm. Hence the increments between solutions were 882, 2343, 5608, 9831, 17,823, respectively, and the sodium influxes would be proportional to the increments of the counts.

Handling does not affect the rate of uptake to a significant extent. Fish placed repeatedly in a 1 mM NaCl/L. solution containing ^{24}Na took up sodium at a constant rate.

Drinking experiments

Although Smith's demonstration in 1930 that marine teleosts drank sea water is widely known, his experiments were brief and have practically never been repeated. Smith placed the animal in a medium containing phenol red after ligating the anus and later measured the concentration of phenol red in the gut. The method is open to several obvious objections. If any phenol red is absorbed from the gut the apparent rate of drinking will be reduced. The turbidity of the gut contents limits the accuracy of the method and the ligation of the anus makes the experiment unphysiological. The method is only practicable on fair-sized fish. In an attempt to overcome some of these objections and to develop a method applicable to small fish two methods were tried. In the first inulin was used as a marker for drinking and the whole fish was homogenized after washing to remove surface activity. Even if some of the inulin was absorbed from the gut it would still be assayed by the method adopted unless it had been completely metabolized to CO_2.

This method may give rather high values for drinking as the result of surface contamination of the fish. In the second method ^{35}S-labeled sulphate was used as a marker and the gut alone was assayed. The experiment was limited to 45 minutes to limit uptake from the gut. Preliminary experiments showed that some activity was found in the body, especially the kidneys, indicating some uptake of sulphate from the gut. After two hours in ^{35}S-labeled sea water the activity in the kidneys amounted to 10% that in the gut. Consequently, the values derived by this method will be rather smaller than the real values. The real volume drunk must lie between those given by the two methods. The discrepancy given by the two methods is larger in fresh water than in sea water. This might be due to the fact that any fresh water drunk will be more extensively and rapidly taken up from the gut, while sea water will be taken up more slowly and less completely.

Three solutions, sea water, 40% sea water and 1 mM/L. NaCl, were made up to contain from 10 to 30 μc. ^{14}C inulin per 100 ml. To the active inulin was added 10 times its weight of inactive carrier but the total concentration of inulin never exceeded 0.04%. Fish were placed in the labeled solution for one hour, removed, washed for 5 minutes, and homogenized in 20 ml. of distilled water and 0.5 ml. sea water. The homogenate was centrifuged at 55,000 RPM for 5 minutes and 0.1 ml. of the supernatant was added to 1 ml. distilled water and 15 ml. of Bray's solution (Bray, 1960). The activity was measured on a Packard Tri-Carb liquid scintillation counter. A standard was prepared by homogenizing an inactive freshly killed fish in 20 ml. distilled water and 0.5 ml. of the original drinking solution. This homogenate was also centrifuged and the activity of 0.1 ml. of the supernatant was measured with the liquid scintillation counter. Surface contamination was corrected by allowing a fish to drink for 5 minutes in the labeled 1 mM NaCl solution, treating as above, and calculating the cpm due to surface contamination by using simultaneous equations. The correction for contamination proved to be only 1% of the original cpm.

Preliminary experiments were carried out in which the fish were placed in active solutions for five minutes, 30 minutes, one hour, two hours and four hours. When allowance was made for the surface contamination the activity was found to increase linearly for the first two hours, after which it showed signs of leveling off. This is interpreted as meaning that swallowed inulin begins to be lost from the anus after about two hours. Measurements were therefore made after one hour of drinking. This obviates the need to ligate the anus.

Sulphate

Sea-water and fresh-water solutions were prepared, each containing 100 μC. ^{35}S-labeled Na_2SO_4 in 250 ml. Fish were placed in a solution for 45 minutes, then removed directly to a washing solution containing MS 222. The anaesthetic was added to reduce the possibility of regurgitation into the pharynx during killing and dissection, which was observed in non-anaesthetized fish. When the fish was immobilized the gut was removed and dropped into 2 ml. of 0.01 N Na_2SO_4 after which it was weighed and then chopped into short pieces about 3 mm. long to allow ^{35}S sulphate to diffuse out. After 6 hours of soaking with frequent shaking the samples were centrifuged and 0.5-ml. aliquots were added to 15 ml. of Bray's solution and counted as before.

RESULTS

Total body sodium, potassium and chloride are given in Table I. It can be seen that while total body potassium remains remarkably constant in the different media, fresh-water fish contain barely half as much sodium and chloride as the sea-water ones.

Total exchangeable sodium

The total exchangeable sodium, calculated from the activity in seven specimens of *Fundulus,* after loading for 24 hours in sea water and washing for 5 minutes in inactive sea water, was 85.3 ± 4.0 m*M* Na/kg. The average exchangeable sodium determined in this way is slightly greater than the total sodium as measured by flame photometry (Table I) although the excess is only equal to the sum of the standard errors. Some of this activity may be due to the activity in the surface mucus not represented in the photometric analyses, which were made on animals whose surfaces had been dried with tissue paper.

TABLE I

Sodium, potassium and chloride content of Fundulus heteroclitus in various media, mM/Kg.

	Na^+	K^+	Cl^-
Sea water	78.7 ± 3.3 (12)	85.2 ± 3.7 (6)	61.3 ± 1.4 (12)
40% sea water	60.6 ± 1.8 (9)	—	50.8 ± 1.2 (12)
Fresh water	48.5 ± 1.5 (10)	80.9 ± 0.7 (6)	31.3 ± 1.8 (12)

Mean ± S.E. (No. of determinations).

After loading for 48 hours or more the activity in the fish declines after transfer to inactive sea water as the sum of two exponentials. If the activity in the fish after time T is A_{ft} then

$$A_{ft} = A_1 e^{-k_1 T} + A_2 e^{-k_2 T}$$

In three fish in which effluxes were measured over a period of 10 hours the average value of A_1 was 0.93 and that of A_2, 0.07 The average rate constant for the fast phase (k_1) was 0.55 h^{-1} and for the slow phase (k_2) was 0.16 h^{-1}. In similar experiments with bromide, A_2 averaged only 0.042 and k_2, 0.028 h^{-1}. Long-term efflux experiments could not be performed with chloride-36 as the sensitivity of the apparatus to beta particles was so low that the counts soon became insignificant.

Rates of efflux

The rate constants of the efflux of sodium, chloride and bromide were determined in each of the three media. The results are given in Table II. The fluxes of both sodium and chloride in fresh water are less than one-twentieth of the fluxes in sea water. The sodium fluxes in 40%, however, were peculiarly variable. This will be discussed further below, but briefly it appears that in this medium some of the fish were behaving approximately as fresh-water fish while others were still behaving as sea-water fish. The median rate constant for sodium in 40% sea water was about 0.09 h^{-1} but the average (Table II) has been weighted by a few larger effluxes. It is clear that the rate constants of the chloride fluxes are rather larger than those of the sodium fluxes, as might be expected with a smaller ion. Bromide is not a good indicator for chloride as it moves more slowly than chloride in both sea water and 40% sea water. In fresh water the rate of bromide efflux is apparently slightly faster than that of chloride, although the data are too limited to decide whether this difference is significant.

Influxes

When the fish are in equilibrium the influx should be equal to the efflux. In fresh water and in 40% sea water the computed influx and efflux rate constants were similar (Table II), but in sea water the influx rate constants appear to be significantly smaller than the efflux rate constants. As explained above, the efflux rate constant is increased by the predominance of the fastest component during a short load, while the influx rate constant is reduced as the function $A_{fx} - A_{ft}$ in equation (2) approximates to the slowest component. The total flux across the body wall may be calculated from the rate constant of the influx multiplied by the total ion content of the animal. In sea water the influx is 20.5 ± 1.8 m*M* Na/kg./hr.: in 40% sea water it is 9.3 ± 2.6 m*M*/kg./hr. and in fresh water only 0.58 ± 0.02 m*M*/kg./hr. The true fluxes are probably rather larger than these for the reasons given above. The flux in sea water is more than 30 times as great as the flux in fresh water.

TABLE II

Rate constants of sodium, chloride and bromide fluxes through Fundulus heteroclitus in various media, h^1

EFFLUX	Na^+	Cl^-	Br^-
Sea water	0.462 ± 0.024 (19)	0.874 ± 0.040 (12)	0.429 ± 0.027 (9)
40% sea water	0.135 ± 0.027 (14)	0.146 ± 0.015 (9)	0.087 ± 0.007 (27)
Fresh water	0.017 ± 0.003 (16)	0.031 ± 0.004 (11)	0.037 ± 0.004 (14)
INFLUX			
Sea water	0.261 ± 0.020 (8)		
40% sea water	0.154 ± 0.043 (7)		
Fresh water	0.0120 ± 0.003 (8)		

Means ± S.E. (No. of determinations).

Rate of drinking

The rate of drinking in sea water as measured by inulin averaged 2.30% body wt./hr., but only 1.54% as measured by sulphate. The real value must lie between these two figures. This is several times as large as reported by Smith (1930) or Motais and Maetz (1964) but their experiments were performed on animals weighing several hundred grams. Drinking continues almost unchanged in 40% sea water although the results in this medium were very variable, ranging from 0.51% to 5.2%. In fresh water the rate of drinking was much lower but was still significant (Table III).

Entry of ions

Salts may enter the fish either through the gut or directly through the body surface. As a result of his experiments, Mullins (1950) believed that the gut was the major or only port of entry in *Gasterosteus* and consequently House (1963) made a similar assumption in interpreting his work on *Blennius*. It is clear that this is not the case in *Fundulus*. In sea water the rate of swallowing is equivalent to only 9.6 μM Na/gm./hr., even if we accept the high result of the inulin experiments: in 40% sea water the rate of swallowing is equivalent to only 3.4 μM/gm./hr. It follows that in all media the greater part of the influx takes place directly through the body surface. Motais and Maetz (1964) found that this was true also in the flounder. The most probable site of this influx is the gills. It is unlikely that there is much influx through the skin, scales or fins, which are poorly vascularized.

TABLE III

Rates of drinking of medium by Fundulus heteroclitus in various media; % body wt./hr.; 20°C.

	^{14}C Inulin	^{35}S Sulphate
Sea water	2.30 ± 0.27 (10)	1.54 ± 0.21 (14)
40% sea water	2.00 ± 0.43 (14)	
Fresh water	0.83 ± 0.39 (7)	0.14 ± 0.05 (14)

Mean S.E. (No. of determinations).

Routes of efflux

The rate of efflux of sodium in sea water and 40% sea water was determined in two groups of fish in which the ureters were ligated. In each group the average rate of efflux was higher than in normal animals. In sea water, $K_0 = 0.462 \pm 0.024$ (19) for normal animals and 0.610 ± 0.06 (11) for ligated animals. Similarly in 40% sea water $K_0 = 0.135 \pm 0.027$ (14) and 0.217 ± 0.053 (9), respectively. This implies that the anaesthetic and the shock of the operation combine to increase the permeability of the body wall to an extent sufficient to mask any reduction of efflux caused by the absence of urine.

Rate of adaptation to fresh water and the effect of fresh water on the rate of efflux

The rate of loss of sodium in fresh water was so low compared with that in sea water that the process of adaptation was investigated in greater detail.

On transfer from sea water to fresh water the rate constant of sodium efflux dropped from *ca.* 0.4–0.6 h^{-1} to *ca.* 0.02–0.05 h^{-1} in about 15 minutes. When transferred back to sea water the efflux remained close to the fresh-water level for about four hours and only returned to the sea-water level after about 18 hours (Fig. 1). This marked asymmetry in the effluxes during transfer from sea water to fresh water and from fresh water to sea water shows that exchange diffusion does not

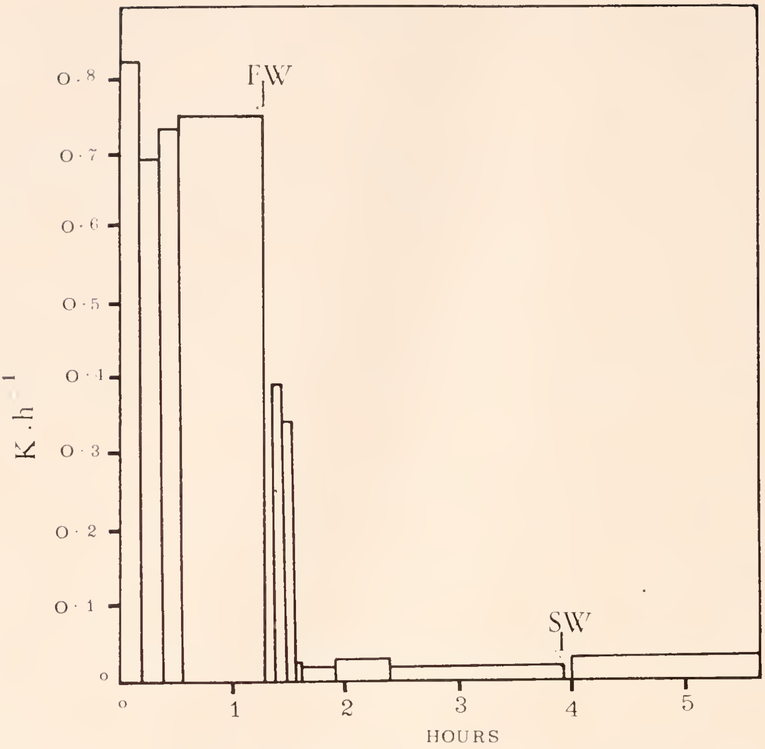

FIGURE 1. Rate constant of sodium efflux in sea water and fresh water. At 1 hour 17 minutes fresh water was run through the bath for a few minutes to remove traces of salt. Similarly at 3 hours 55 minutes sea water was run through the apparatus. At these times no measurements were possible.

contribute substantially to the rate of efflux in sea water. However, it might be argued that the high efflux was due, in the main, to the active extrusion of sodium consequent on the drinking of sea water in order to maintain water balance. After several hours in fresh water the fish might become water-loaded and not start to drink for some time after returning to sea water. To examine this possibility sea-water-adapted fish were transferred to fresh water for limited periods of time, then loaded in active sea water for two hours and the rate of efflux in sea water was then measured during the succeeding two hours. It was found that only 10 minutes in fresh water reduced K_0 to 0.285 ± 0.08 *(7)* while 15 minutes reduced K_0 to 0.190 ± 0.07 (8).

DISCUSSION

The regulation of the total body salt after transfer between media seems at first sight to be very poor. Black (1948) found an even greater variation in chloride content between sea-water- and fresh-water-adapted *Fundulus* (sea water 55 m*M* Cl/kg., fresh water 20 m*M* Cl/kg.) but the low value in fresh water may be related to the very low concentration of chloride (0.07 m*M*/L.) in her fresh water. Burden (1956) found that the chloride concentration of the blood in sea water was not significantly different from that in fresh water, while Pickford (1965) has found only a small decline in total osmotic pressure (sea water = 180 m*M*/Cl/L., fresh water = 162 m*M*/NaCl/L.) but very recently Motais *et al.* (1966) report that the plasma sodium of *F. heteroclitus* declines from 193 m*M*/L. in sea water to 156 m*M*/L. in fresh water. Some part, at least, of the discrepancy between the variable total body sodium and the more constant plasma concentration may be attributed to the high concentration of salt in the gut of sea-water-adapted fish. Motais (1961) and Motais and Maetz (1964) have shown that the sodium in *Platichthys* gut exchanges only slowly with the sodium in the blood.

A large reduction of the sodium and chloride fluxes on adaptation to fresh water has also been found in many teleosts. The sodium flux through *Gasterosteus* was reduced from 11 m*M*/kg./hr. in a dilute Baltic sea water to 0.7 m*M*/kg./hr. in fresh water (Mullins, 1950). In *Platichthys* the rate constant of sodium exchange declined from 0.18 to 0.3/hr. in sea water to 0.01/hr. in fresh water (Motais, 1961), while Gordon (1963) showed a similar reduction in chloride fluxes in *Salmo*. However, Mullins (1950) found that in sticklebacks (*Gasterosteus*) adapted to Baltic sea water, containing only 267 m*M* Cl/kg. water, or 9.5% chlorinity, the rate of uptake of several ions, sodium, potassium, bromide and phosphate, was similar when expressed in the terms

$$\frac{\text{Activity/gm. fish}}{\text{Activity/gm. solution}}$$

Mullins suggested that this result could be accounted for by the assumption that the fish drank the equivalent of 4% of their body weight/hr., but in the light of later work the result must be attributed to a coincidence. Gordon (1963) has measured the rate of efflux of chloride from both normal rainbow trout, *Salmo gairdneri,* and from trout with ligated cloacae. The efflux from trout adapted to sea water was between 10 and 20 times the efflux from trout in fresh water but the variations between the small numbers of individual fish used and between fish at different times of the year makes any conclusions hazardous. Fish with ligated cloacas had an average rate of efflux in sea water about one-third of that of normal animals. Gordon suggested that the renal loss in sea water exceeded the extrarenal loss but this is impossible under the Smith hypothesis of osmoregulation in marine teleosts. The urine of marine teleosts is approximately isosmotic with the blood and contains a high concentration of divalent ions. Holmes (1963) points out that for every liter of sea water drunk the corresponding urine flow must be less than one liter and the sodium chloride loss in the urine can only be equivalent to a small part of the total taken in by drinking. Motais *et al.* (1966) have demonstrated that a rapid decline in efflux takes place in a variety of euryhaline teleosts on transfer from sea water to fresh water. In *Platichthys* a large part of the exchange in sea water is due to exchange diffusion but in *Fundulus* the exchange diffusion component is small, *ca.* 15%. The exchange diffusion component causes an instantaneous reduction in the efflux on transfer to fresh water but in both species a reduction takes place in the permeability of the fish to ions in the hours following transfer which causes a further reduction in efflux.

Practically no direct measurements of drinking rates have been published since Smith's original measurements. Smith estimated the rate of drinking from the concentration of dye in the gut of fish, with ligated cloacas, kept in sea water containing phenol red. He estimated that eels, *Anguilla,* and sculpins, *Myxocephalus,* drank between 50 and 200 ml./kg./day, average 60 ml./kg./day, of which three-quarters was absorbed from the gut. The residual fluid was iso-osmotic with the blood and contained mainly divalent ions. Smith suggested that the salt absorbed is excreted extra-renally as a hyper-osmotic solution but this would entail further water loss. It is likely that the ions are secreted directly into the sea water. Keys (1931) and Schlieper (1933) showed that salt was secreted from the anterior end of the body, and it is probable that the gills are the site of secretions although the identity of the cells responsible has been the subject of a long controversy.

Motais and Maetz (1964), using Smith's original technique, estimated that *Platichthys* drank 0.73–1.37% body wt./hr. (mean 1.0% hr.), equivalent to an influx of 5 m*M* Na/kg./hr. The total sodium flux averaged between 10 and 12.5 m*M*/kg./hr. Here also the greater part of the influx took place outside the gut. Drinking evidently continues in iso-osmotic sea water, and at a reduced rate, even in fresh water. Frank and Allee (1950), using colloidal thorium dioxide as a marker, also found that *Fundulus diaphanus* continued to drink in fresh water although Bergeron (1956), using colloidal carbon, could detect no drinking in fresh water in *F. heteroclitus.* However, Bergeron was able to detect drinking in solutions as dilute as $\Delta 0.04^\circ$ C. = 10 m*M* NaCl/L. Neither Bergeron nor Frank and Allee made any quantitative estimates of drinking. In sea water the total influx of sodium amounts to 20.5 m*M*/kg./hr. Less than one-half of this enters by the gut, while the rest, 11 m*M*/kg./hr., must enter through the body surface, especially the gills. Until the chloride fractions have been analyzed in detail it is not possible to define the chlorine fluxes exactly but from Table II it seems likely that they too are similar but somewhat greater than the sodium fluxes. A drinking rate of 2.3% per hr. corresponds to an influx through the gut of 11.3 m*M* Cl/kg./hr. while the total chloride influx may be of the order of 40 m*M*/kg./hr. It is probable that the passive chloride influx is proportionately greater than that of sodium as the sea-water chloride concentration is higher and the chlorine ion smaller than the sodium ion.

In 40% sea water the rate of drinking as measured by inulin corresponds to a sodium influx of 3.4 m*M*/kg./hr., again less than half the total. The passive influx must be about 5 m*M*/kg./hr., suggesting that the permeability to sodium in this medium is similar to that in sea water. In fresh water the passive influx is almost negligible and nearly all the influx must be due to active uptake. This varies with the external concentration in a manner similar to that found in the fresh-water Crustacea (Shaw, 1959, 1961; Shaw and Sutcliffe, 1961). The relationship between active influx, f, and external concentration, M, may be represented by the equation

$$f = f_{\max}\left(\frac{M}{M + C}\right)$$

This equation is similar in form to the Michaelis-Menton equation relating the rate of an enzymatic reaction to the concentration of substrate. In the influx equation, C is the concentration at which $f = \frac{1}{2} f_{\max}$ and is a measure of the affinity of the carrier molecule to the ion. In general the lower the value of C, the lower the concentration in which the animal can survive. In *Fundulus*, C is about 2 m*M* Na/L. In fresh-water Crustacea values of C are found as low as 0.2 (*Astacus*) or 0.1 m*M*/L. (*Potamon*). On this evidence *Fundulus* is not as well adapted to fresh water as these crustaceans and indeed *Fundulus* does not survive well in tap water.

Sodium loss may occur in several ways: by diffusion through the body wall, in the urine and from the gut. In fresh-water fish the rate of urine production is usually less than 1% per hr. and the urine is hypo-osmotic to the blood, so that the renal loss of sodium is small. Meier and Fleming (1962) have shown by ligation experiments that in the plains killifish, *F. kansae*, 28% of the total sodium loss takes place through the kidneys, 10% through the gut and the rest through the body surface. Stanley and Fleming (1964) found that in fresh water *F. kansae* produced 8.3 ml./kg./hr., containing an average of 12.8 m*M* Na/L. The sodium loss would amount to 0.01 m*M*/kg./hr.

If we assume a similar distribution of losses in *F. heteroclitus* the sodium loss through the body surface in fresh water will be reduced to *ca.* 0.36 m*M*/kg./hr. The chloride flux across the body wall may be independent of the sodium flux when the animal is in equilibrium.

The losses in sea water are more difficult to evaluate since a small passive efflux of sodium will occur even in this medium. The inward diffusion in sea water is of the order of 11 m*M*/kg./hr. The ratio, passive efflux/passive influx, will be equal to blood concentration/sea water concentration in the absence of any potential difference. The passive efflux in sea water would therefore be about 4 m*M*/kg./hr. if there were no potential difference. Renal loss in sea-water-adapted fish must be small. Urine flows in marine teleosts are of the order of 1–2% body weight/day (Black, 1957; Holmes, 1963). In the related species *F. kansae*, adapted to sea water, the rate of urine production was 0.87 ml./kg./hr., containing 140 m*M* Na/L. (Stanley and Fleming, 1964). The renal sodium loss in sea water was therefore 0.12 m*M*/kg./hr.

Some loss of sodium will take place from the anus. In *Lophius* the sodium concentration of the fluid in the lower end of the intestine was only 56 m*M*/kg. water (Smith, 1930). The volume of fluid lost from the gut is unknown but can only be a small fraction of the sea water drunk. Again in *Lophius* the sulphate concentration at the lower end of the gut was 6 times as great as in the sea water (Smith, 1930), since sulphate is absorbed slowly if at all; thus at least five-sixths of the sea water taken in had been absorbed. It may be concluded that although some sodium will be lost from the rectum it is unlikely to exceed 10% of that taken in through the gut and is likely to be even less. Sodium loss from the rectum in sea water may therefore be estimated at less than 1 m*M*/kg./hr. The remaining sodium loss, *ca.* 16 m*M*/kg./hr., must be due to the active extrusion of sodium against the concentration gradient. Fleming, Scheffel and Linton (1962) have shown that the active extrusion of sodium chloride by marine teleosts is associated with cholinesterase activity in the gill. The efflux of sodium from *F. kansae* is reduced by 75% by treatment with 5×10^{-6} *M* eserine.

Influxes, effluxes and drinking rates are all more variable in 40% sea water than in either sea water or fresh water. Some efflux rate constants in fish adapted to 40% sea water are similar to those of fresh-water fish and others to those of sea-water fish. From this it seems probable that some fish are behaving as sea-water-adapted fish, while others appear closer to fresh-water-adapted fish.

The most striking difference between the sea-water-adapted fish and the fresh-water-adapted fish lies in the permeability of the body wall to ions. It is clear that the most important single change during adaptation to fresh water is the reduction of permeability. An external sodium concentration of 425 m*M*/L. generates an influx of 11 m*M*/L. through the body surface. In fresh water a blood concentration of *ca.* 150 m*M* Na/L. would generate an efflux of *ca.* 4 m*M* Na/L. if the permeability remained unchanged and there was no potential across the body wall, but the observed efflux is about one-tenth of this. If this reduction in permeability did not occur, survival in fresh water would only be possible if the rate of active uptake were increased many times. As was shown in an earlier paper, hypophysectomized *Fundulus* in fresh water fail to survive, in the absence of prolactin, because their permeability does not remain high. Normal fish which have been adapted to fresh water or even placed in fresh water for a short period, maintain a low permeability for many hours after return to sea water (Fig. 1). The rate of turnover of sodium is greatly reduced between two and four hours later after only 15 minutes in fresh water. The rapid, but not instantaneous, adaptation and slow reversal suggest that the permeability is controlled by a hormone released in response to fresh water and/or a falling blood concentration. The exchange diffusion component in the sea water flux is small.

While the advantages of a low permeability to ions in fresh water are obvious, the advantages of a high permeability in sea water are not. Marine fish must work to maintain osmotic equilibrium in sea water and reduction in permeability would reduce this work. The increased permeability observed in sea-water-adapted fish presumably confers some advantage but no convincing suggestion can be offered.

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SUMMARY

Measurements have been made of the rates of influx and efflux of sodium ions in sea water, 40% sea water and fresh water in the killifish, *Fundulus heteroclitus.* Measurements have also been made of the rates of efflux of chloride and bromide ions in the same media. The rate of drinking has been measured using inulin-labeled sea water. In sea water the sodium influx averages 20 m*M* Na/kg./hr., of which more than half enters by diffusion, the rest by drinking. In fresh water the rate of influx is about 0.6 m*M* Na/kg./hr., practically all of which takes place by active transport. Adaptation to fresh water is accompanied by a great reduction in permeability to sodium and chloride ions. This fall in permeability takes place within a few minutes of transfer to fresh water but the increase of permeability on return to sea water takes many hours. The relationship between active uptake and external concentrations at low concentrations resembles that found in fresh-water crustaceans.

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MORPHOGENESIS OF EPITOKOUS SETAE DURING NORMAL AND INDUCED METAMORPHOSIS IN THE POLYCHAETE ANNELID NEREIS GRUBEI (KINBERG)[1]

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Most species of nereid polychaetes end their lives by undergoing a complex metamorphosis in preparation for a single brief period of reproductive activity. The metamorphosis is completed at the time of sexual maturity. At this time, having acquired modifications which adapt them to a pelagic existence, these formerly benthic animals leave their mucus tubes and swarm in midwater, where mass spawning takes place.

Although the production of paddle-shaped "pelagic" setae is one of the most characteristic features of nereid metamorphosis, the origin of the chaetogenic tissue has not yet been completely described. The characteristic setae of the benthic atoke are replaced throughout the posterior region with an orderly array of heteronereid setae. These greatly increase the effective surface area of the parapodium and probably serve as oars during the brief pelagic existence of the mature worm. Heteronereid chaetogenesis has been described in the European *Perinereis cultrifera* by Bauchot-Boutin and Bobin (1954). The author has observed a similar series of events in *Nereis grubei*, but the observation of earlier stages now requires a new interpretation of the origin and nature of the chaetogenic tissue.

Nereid metamorphosis is known to be regulated by an inhibitory hormone produced by cells in the supra-esophageal ganglion (Durchon, 1952), but no attempt to define the response of individual somatic tissues to hormone deprivation has yet been reported. The information reported here on the earliest stages in the process of chaetogenesis permits an accurate interpretation of the response of parapodial epidermis to the removal of the inhibitory hormone at different stages of normal development.

Materials and Methods

Specimens of *Nereis grubei* were collected from burrows in the holdfasts of the brown kelp *Egregia menziesii* and from the sandy substrate beneath the abundant red alga *Gasteroclonium coulteri* at Pescadero Point, Monterey County, California.

Control animals were sacrificed immediately after oöcyte measurements had been taken. Experimental animals were maintained in deep Petri dishes in filtered, antibiotic-containing sea water (terramycin, 25 mg./l. or a combination of penicillin, 10^6 units/l. and streptomycin, 100 mg./l.). The temperature was maintained at 15–16° C. by flow of cool sea water around the bases of the Petri dishes.

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Oöcyte diameters were determined by removing a sample of coelomic contents from about segment 30 of worms anesthetized in 7.3% $MgCl_2$. Each sample was removed in a disposable capillary micropipette, deposited in a drop of mineral oil on a circular coverslip, and examined as a hanging drop preparation. Diameters of 10–15 oöcytes, including all sizes present, were recorded for each animal, and in those cases where the oöcytes present appeared to include two distinct size classes, the average diameter of the largest size class was used to characterize the animal (Schroeder, 1966). In instances where two size classes were not apparent, all the oöcytes from a given animal were averaged. The diameters were all measured from living cells with the same binocular microscope.

Rates were determined by sequential measurements from animals maintained in the laboratory without feeding.

Decapitation involved the removal of the prostomium and the peristomial tentacles from anesthetized animals with fine Wecker scissors. In most cases the proboscis of the experimental animals was not injured and the wound healed well. Previously anesthetized animals were fixed in sea water Bouin's fluid. After fixation, segments 44–46 were cut from each specimen, imbedded in 61° paraffin and sectioned at 5–8 μ. Although no autoradiographic observations are reported here, most of the slides upon which this study is based were autoradiograms of serial cross sections. The slides were dipped in Ilford K5 Liquid Nuclear Emulsion, exposed for 2–4 weeks in a dark, dry atmosphere, developed in Kodak D-19 and stained with Mayer's hemalum and celestine blue (Doniach and Pelc, 1950). Some slides were stained with Ehrlich's hematoxylin and triosin, Mallory's triple stain, and Heidenhain's Azan procedure.

The terms "chaeta" and "seta" have been considered synonymous, and are used interchangeably herein. "Seta," the Latin form, has been preferred as a noun; "chaeta," the Greek form, has been preferred in combinations.

Morphogenesis of Heteronereid Setae

The appearance of the tissue which will ultimately produce the heteronereid setae is among the earliest detectable events of metamorphosis. Its initial formation occurs when the major oöcyte size class reaches a diameter of approximately 95 μ. The subsequent development of the chaetogenic tissue may be divided into four stages, which are summarized in Table I. Separation of the first three stages

Table I

Stages in the development of the heteronereid setal sacs

Stage	Characteristics	Oöcyte diameter	Duration*
I	Movement of proliferated epidermal cells along atokous setal sac; differentiation of first chaetoblast cells	95–130 μ	*ca.* 22 days
II	Appearance of paddles within the chaetogenic tissue	130–165 μ	*ca.* 22 days
III	Appearance of setal shafts within the chaetogenic tissue	165–180 μ	*ca.* 9 days
IV	Eruption of fully formed setae to the exterior	over 180 μ	*ca.* 13 days

* Durations are calculated from an oöcyte growth rate of 1.6 μ/day and are approximate. The duration of stage IV is especially likely to be variable. (See text.)

depends upon observation of the characteristic sculpturing of different portions of the heteronereid setae with phase contrast optics (Fig. 1). The first stage includes the period of proliferation and is terminated at about the time that the oöcytes reach a diameter of approximately 130 μ. At this point, about 22 days after the initiation of stage I, the first signs of the paddles (the terminal portions of the compound setae) make their appearance within the chaetogenic tissue. Stage II comprises the period during which only the paddles may be detected and also lasts about 22 days. When the oöcytes are 165–170 μ in diameter, the shafts appear within the chaetogenic tissue. The presence of these shafts defines stage III, which lasts until the paddle-ends erupt through the epidermis to the exterior, a period of about 9 days. After eruption, which occurs when the oöcytes are approximately 180 μ in diameter, the setal sacs are in the final stage IV, where they remain for approximately two weeks while metamorphosis is completed. Since stage definition rests upon unambiguous morphological criteria, the stage of any metamorphosing specimen may be accurately determined, even from cleared and stained whole mounts of parapodia.

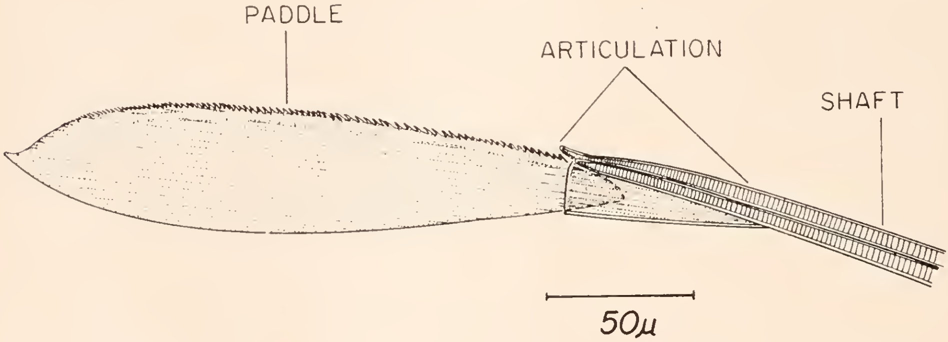


FIGURE 1. The structure of a heteronereid seta, illustrating the characteristic sculpturing of the components.

Setal sac development was found to progress somewhat independently in the dorsal and ventral portions of the parapodium (the notopodium and the neuropodium, respectively). The notopodium is occasionally found to be in a stage slightly more advanced than the neuropodium; and the slightly more rapid development of the heteronereid setae in the notopodium is probably a regular feature of setal sac development.

Stages in setal sac morphogenesis

Stage I

New chaetogenic tissue appears to arise by proliferation from the epidermis at two sites on the parapodium of the atoke. These areas are indicated in Figure 2 at the bottom of deep folds just dorsal to the notopodial setal bundle and ventral to the neuropodial setal bundle. The tissue is especially prominent in autoradiograms stained with Mayer's hemalum and celestine blue. Proliferating cells spread over the surface of the atokous setal bundles, which are separated from the coelom by an aniline blue-positive peritoneum. Peritoneal nuclei may also be seen lining the coelomic surface of the new chaetogenic tissue, which inserts itself between the atokous setal sac and its peritoneal covering. The most characteristic cells of the new tissue, thought to represent chaetoblasts, have large nuclei with one to three prominent nucleoli. With the stain used, the boundaries of the cells could not be distinguished, and Figure 2 shows the tissue as it appears in both the notopodium and the neuropodium.

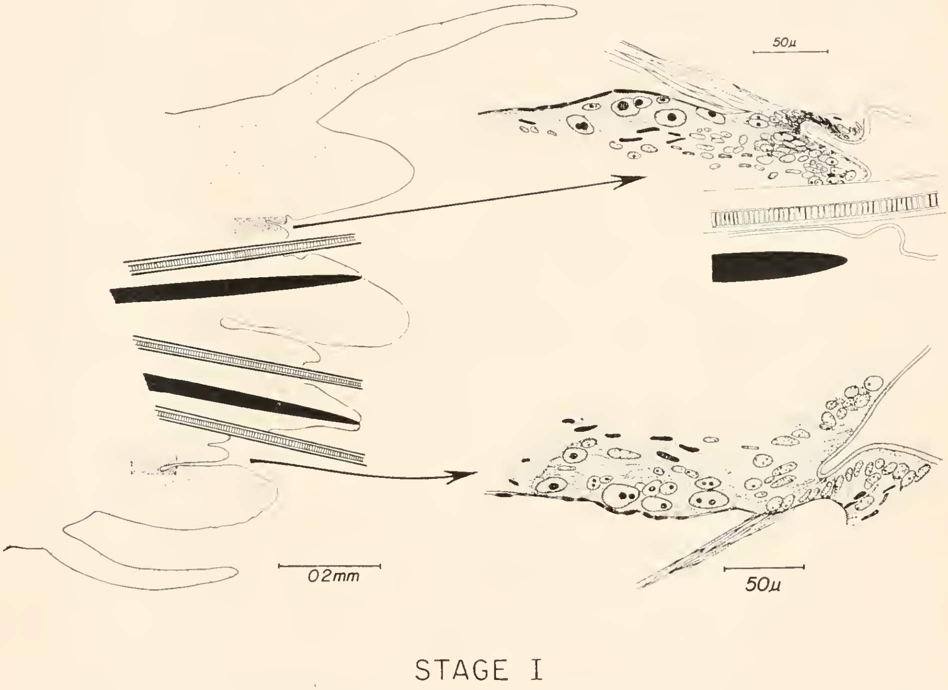
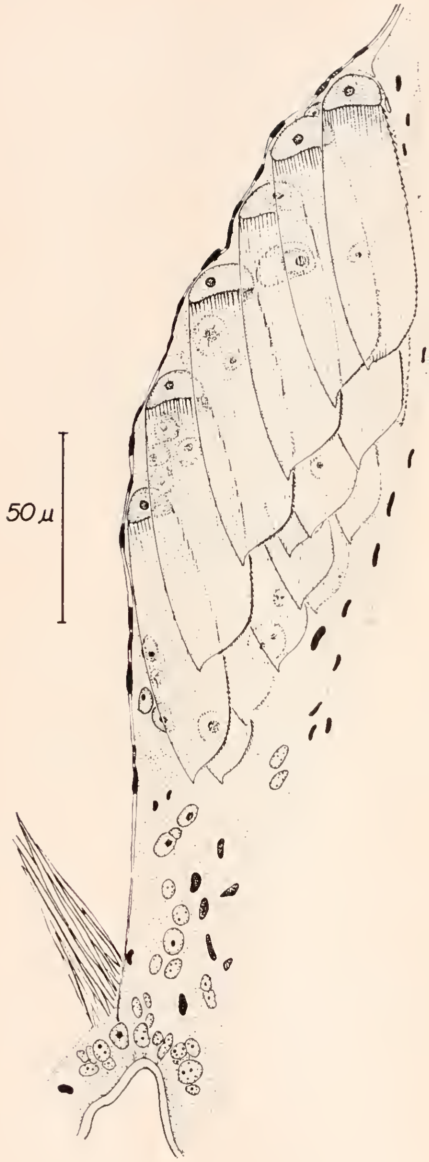


Figure 2. An outline of an atokous parapodium. The chaetogenic areas for the notopodial and neuropodial heteronereid setae are stippled. Insets: the appearance of the earliest stage of development of the heteronereid setae. Upper: stage I, notopodium. Lower: stage I, neuropodium.

Setal sacs in stage I can be detected in parapodial whole mounts. After staining with an alum hematoxylin and clearing in toluene, the new tissue appears as a pair of basophilic mounds on the dorsal surface of the notopodial setal sac and the ventral surface of the notopodial setal sac.

Stage II

Stage II is defined by the presence of paddles, which are the blade-like terminal portions of the heteronereid setae. Figures 1 and 3 show the characteristic fine striations which parallel the longitudinal axis of the blade. These are best seen



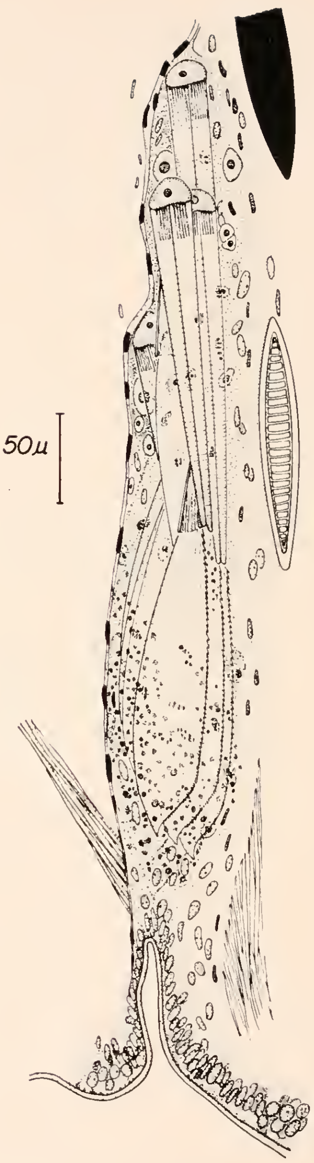


FIGURE 3. Diagrammatic longitudinal section of a neuropodial setal sac in stage II.

FIGURE 4. Diagrammatic longitudinal section of a neuropodial setal sac in stage III. A cross section of an entire worm yields longitudinal sections of the parapodia, from which these diagrams were prepared.

with phase contrast optics, which permit the detection of even small portions of paddles in unfavorable sections. The paddles are secreted by prominent chaetoblast cells at the base of the developing setal sac. The sculpturing appears to be molded by a well-developed "ciliary" border (*appareil ciliaire*) along the secretory edge of the chaetoblast (Bauchot-Boutin and Bobin, 1954), which is indicated in Figure 3 by thickened lines of sculpturing at the base of the paddle. The paddles are stacked closely together, but stainable material and occasional prominent nuclei are present between them. A tenuous connection with the epidermis is retained. Although no further cells are contributed to the setal sac once paddle production is underway, the connection may be identified by its greater basophilia.

Stage III

The appearance of the shafts, with their characteristic double column sculpturing at right angles to the setal axis (Figs. 1 and 4), provides a convenient and unambiguous criterion for the determination of stage III. The shafts of the atokous setae are sculptured with but a single column of short horizontal bands (Figs. 2 and 4). The presence of two such columns in the shaft of the heteronereid seta provides a ready distinction between the two types in sections, even when the terminus of the setae is lost. Near the articulation of the atokous setae the columns are occasionally multiplied, so that comparison must be made between portions of the shaft removed from the articulation.

The paddles reflect light brilliantly, and, when well-developed, may be seen within the parapodia of living specimens. Stages II and III may not be distinguished, however, without examination of an isolated parapodium under higher magnification.

Stage IV

Stage IV is also defined by an unambiguous event, the eruption of the paddle ends through the parapodial epidermis. The point of eruption is readily found in a series of sections. It usually occurs when the oöcytes are about 180 μ in diameter, and swarming is estimated to follow in about 13 days.

It seems probable that the initiation of stage I is coincident with the increase in the growth rate of the oöcytes. The average rate of growth of oöcytes over 100 μ in diameter is about 1.6 μ/day, and is distinct from the much slower growth rate of smaller oöcytes (Clark and Ruston, 1963; Schroeder, 1966). Since the range of oöcyte diameters is known for each stage, the duration of each stage has been calculated and included both in above description and in Table I. These values add to a total of 67 days for metamorphosis of the setal sacs and thus perhaps for the interval between the first waning of the level of the inhibitory hormone and actual swarming. Specimen G135, taken from the field with 111 μ oöcytes, swarmed 62 days later in the laboratory. Using the total given above, corrected for the late start, swarming would have been predicted after 58 days.

It should be recalled that the above timetable applies to segments 44–46. The timetable of setal sac morphogenesis, and especially its correlation with oöcyte growth, varies along the length of the heteronereid region (Reish, 1954).

Effects of Decapitation on Setal Sac Development

Removal of the prostomium deprives nereids of the hormone which inhibits metamorphosis, and therefore induces metamorphic changes prematurely. An analysis of the relationship between the development of the setal sacs and the presence of the hormone has been possible because of the well-defined stages into which the process can be divided, and because information on the oögenesis of the species is available (Schroeder, 1966).

Figure 5 illustrates normal setal sac development in relation to oöcyte growth, and the range of oöcyte diameters found during each normal setal sac stage is indicated. The normal duration of each setal sac stage has also been indicated by the stippled areas on the graphs (Fig. 5) of the experimental results, to facilitate their comparison with normal development. Since the average oöcyte growth rate is known, this figure has been used to correct the data presented for operated animals of oöcyte diameter greater than 95 μ. Thus when an animal with 100 μ oöcytes is decapitated and maintained for 22 days, it can be predicted that with normal growth the oöcytes would have been approximately 135 μ in diameter at the end of the 22 days, and that correspondingly the setal sacs ought to be in stage II. They are in fact found to be in stage IV, and their development has

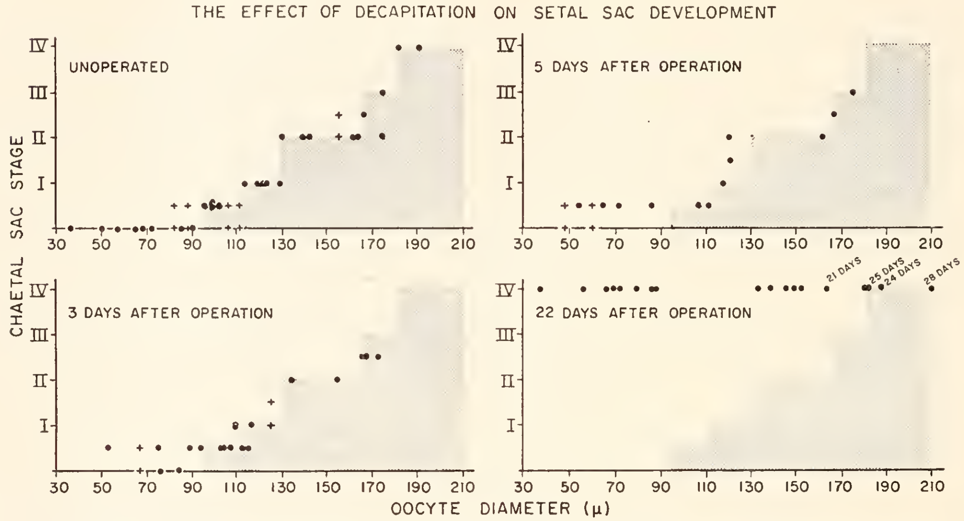


Figure 5. The development of the heteronereid setal sacs and their response to decapitation. For specimens in which the notopodium is in a slightly more advanced stage than the neuropodium, the point for the animal has been split into two crosses, the upper of which designates the neuropodium in all cases. In order to give a clearer picture of development, animals which had obviously just entered upon a given stage are reported as being midway between the two stages on the graph. The periods of normal development have been indicated on the graphs of the experimental results to facilitate their comparison with normal development. The points for experimental animals with oocytes over 95 μ original diameter have been corrected as described in the text.

clearly been accelerated. Such corrections have not been made for animals with oöcyte diameters under 95 μ since the absolute value of the oöcyte growth rate in these animals is less well established. It should also be noted that in some young animals a few deeply basophilic cells are present which represent the earliest appearance of stage I. These animals have been represented in the graphs as a partial stage I, since they include many fewer cells than are illustrated in Figure 2. In a similar manner, the earliest appearance of the paddles has been noted as a partial stage II, and the earliest appearance of the shafts as a partial stage III, in order to provide a better picture of the progress of development.

The effect of decapitation upon the development of the heteronereid setae has been examined in a series of animals of increasing age. Decapitation of gameteless animals induced the formation of a few small, relatively unsubstantial heteronereid setae. The animals were examined 15 or more days after decapitation, and 11 of 12 animals had produced the tiny "pelagic" setae. Since the setae thus unnaturally produced are smaller and more transparent and not as readily found as normal heteronereid setae, it is probable that they had been produced but were not observed in the one exceptional individual.

Decapitation of animals with oöcytes under 95 μ in diameter, which have not yet begun to develop heteronereid setae, also induces the formation of a few tiny "pelagic" setae. These were observed 22 days after decapitation in 7 out of 8 animals, whose oöcytes ranged from 37 μ to 88 μ in average diameter. Additional animals with oöcytes under 95 μ were examined 3 and 5 days after decapitation (Fig. 5). In 4 of the 6 animals examined 3 days after decapitation recognizable chaetoblast cells were present, although not as many as in the full stage I (Fig. 2). After 5 days, such chaetoblasts were present in at least one portion of the parapodium of all 6 of the animals examined. No traces of further development were found after 5 days, although after 22 days the differentiation and production of heteronereid setae is complete. Thus, at least a few cells of the parapodial epidermis appear to be competent to differentiate in response to hormone deprivation, even in very young worms.

Results obtained by decapitation of animals with oöcytes greater than 95 μ in diameter and in which normal production of the heteronereid setal sacs has begun, are also reported in Figure 5. The oöcyte sizes indicated for the experimental animals in this oöcyte size range have been corrected to account for normal oöcyte growth. Animals sacrificed 3 and 5 days after decapitation demonstrate very little acceleration of setal sac morphogenesis. Only two of these specimens, both stage I animals sacrificed 5 days after decapitation, appeared to show a response to the operation. The 22-day experiments clearly indicate that decapitation does yield accelerated development when performed on animals in stages I and II. It seems likely that the first two stages last too long to permit the histological resolution of changes induced in them over short periods.

For various reasons, a number of animals was sacrificed at irregular intervals after decapitation, and data from 14 such cases are summarized in Table II, arranged in the order of increasing oöcyte diameter. For those with oöcyte diameters above 95 μ, estimates of normal oöcyte growth and setal sac stage for the post-operative period involved are included. Acceleration of setal sac development is clearly shown in animals with oöcytes up to 140–145 μ, even after only

TABLE II

Decreasing response of setal sac development to hormone deprivation during metamorphosis

Animal no.	No. of days	Average oöcyte diameter (μ)		Setal sac stage	
		Initial	Expected	Expected	Observed
G375	17	56			Notopodium IV Neuropodium 0
G403	13	108	129	I–II	IV
G153	18	115	138	II	IV
G188	7	130	141	II	III
G160	8	132	145	II	III
G168	7	138	149	II	III
G241	15	140	164	II–III	III
G165	4	143	149	II	II
G436	13	159	180	III–IV	early III
G437	4	161	167	II–III	III
G207	6	162	172	III	III
G205	5	167	175	III	III
G206	6	169	179	III–IV	III
G458	13	170	191	IV	III

TABLE III

A concordance of stages of post-larval development in epitokous nereid polychaetes

Proposed stage		Author's designation	Characteristic of stage
1		Durchon 0 Hauenschild 3	Atokous, without gametes
2		Charrier A Hauenschild 2	Atokous, with oöcytes growing at slower rate. No heteronereid chaetoblasts present
3	Hauenschild 1	Setal Sac I	Differentiation of first chaetoblasts; increase in oöcyte growth rate; probable decrease in hromone level
—		Durchon I	Shortening of segments; increased vascularization
4		Setal Sac II	Internal appearance of heteronereid paddles
—		Charrier B Durchon II	Enlargement of parapoidal lobes
5		Setal Sac III	Internal production of heteronereid shafts
—		Charrier C	"Heteronereis without swimming setae"
6		Durchon III Setal Sac IV	Eruption of heteronereid setae from parapodia
7		Charrier D Durchon IV Hauenschild 0	Oöcytes with mature morphology; loss of atokous setae; completion of parapodial transformation

seven days. Specimens with larger oöcytes do not show acceleration, and two do not even reach the stage expected. When comparing this table to the results reported in Figure 5 it should be recalled that the oöcyte diameters given there for animals with an initial oöcyte diameter over 95 μ have been corrected, and correspond to the expected oöcyte diameters in Table II. The hormone level is presumably waning during metamorphosis and a point is probably reached at which the hormone level is so low that it no longer influences setal sac morphogenesis.

Stages of Nereid Post-Larval Development

The stages in the development of the heteronereid setae provide a more precise and accurate basis for subdividing the period of nereid metamorphosis than has heretofore been available. The stages previously reported by Charrier (1920) and Durchon (in Bauchot-Boutin and Bobin, 1954) are based upon the appearance of external features which cannot be defined with precision. Table III is an attempt to correlate the stages described by these authors, the setal sac stages defined herein, and the broader, more physiologically defined stages of Hauenschild (1966), into a series of stages which will define the whole of the post-larval existence of an epitokous nereid. Examination of a female specimen should permit its assignment to any of the seven periods, although the sacrifice of a parapodium may be required to accomplish this most accurately if the oöcytes are sufficiently well-developed. The stages may be applied to males, but the events of spermatogenesis have not yet been correlated with setal sac development.

Discussion

The development of the heteronereid setae was studied by Bauchot-Boutin and Bobin (1954) from cross sections of the parapodia of *Perinereis cultrifera*. Although what they saw in this species does not appear to differ essentially from the observations on *N. grubei*, they did not observe the earliest stage of the process and interpreted the origin of the chaetogenic tissue to be different from what has been seen in *N. grubei*. They concluded that the heteronereid setae were produced from a germinal layer of chaetoblasts located on the external border of the setal sac, which had formerly produced replacement setae for the atoke. They were primarily interested in showing that the changeover to production of the new setal type is accomplished by a mechanism different from that involved in the regeneration of simple setae in sabellids, in which an injured setal sac degenerates and is replaced with new chaetogenic tissue before a replacement seta is produced (Bobin, 1947). The apparent unavailability of the earliest stages of heteronereid chaetogenesis forced them to commence their examination at last in setal sac stage II, since paddles are unquestionably present in their illustration. In fact, the chaetoblasts which are producing these setae do arise from a new epidermal proliferation. Although both nereid and heteronereid setal sacs are present simultaneously, there is a gradual reduction in the volume of the atokus chaetal sac. The vacated space is occupied by the developing heteronereid tissue. Since the animal retains functional atokous setae until quite late in metamorphosis, the atokous tissue is present in a reduced but still functional state until their loss, which occurs completely only during stage IV, after the eruption of the heteronereid setae.

Stage duration

In the calculation of the duration of each of the setal sac stages it has been assumed that the rate of oöcyte growth during each of the two growth phases is constant. However, in *Nereis diversicolor,* Clark and Ruston (1963) found that the rate of oöcyte diameter increase slowed down as the maximum diameter was approached. Although no evidence for such a rate change has been detected in *N. grubei,* the possibility that such a slow-down occurs in this species has not been completely eliminated. In that case, the calculated duration of stage IV, and possibly of stage III, would fall short of the true values. Good agreement of calculated growth rates with the timing of events in an animal which metamorphosed on the sea table lends credence to the calculations for *N. grubei.* It is possible that the different breeding habits of *N. diversicolor* might involve an additional change in oöcyte growth rate not found in epitokous species.

The appearance of heteronereid setae in the parapodia of young decapitated nereids has been reported in *Perinereis cultrifera* (Durchon, 1965) and in *Platynereis dumerilii* (Hauenschild, 1966). Durchon found that only 4 of 38 decapitated four-month-old specimens of *P. cultrifera* produced heteronereid setae. Hauenschild found that the parapodia of decapitated specimens of *P. dumerilii* with oöcytes under 45 μ underwent few externally visible metamorphic changes, with the exception of the occasional appearance of small, thin heteronereid setae. Since the heteronereid setae found in the parapodia of decapitated, gameteless *N. grubei* were recognized only after a careful re-examination of sectioned parapodia, their appearance may be more consistent than the initial reports indicated. The parapodial epidermis is thus competent to differentiate long before the stimulus to do so is normally supplied.

This study reveals that the first stage in heteronereid chaetogenesis occurs at the same time that the oöcyte growth rate increases in normal ontogeny (Schroeder, 1966). This fact supports the assumption that these two phenomena are controlled by the same hormone, which in *Nereis grubei* begins to wane more than two months before metamorphosis is completed and swarming takes place.

It is hoped that the description of these setal sac stages will engender greater precision in future work on nereid metamorphosis and its control, particularly when the response of isolated parapodia is used as an assay procedure.

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SUMMARY

1. The development of the heteronereid setae during the metamorphosis of *Nereis grubei* is described. Four stages may be distinguished by the sequential appearance within the chaetogenic tissue of the several components of these compound setae. The characteristics of each stage are summarized in Table I.

2. Initiation of setal sac development results from the decapitation of even very young gameteless animals. At least a few cells of the parapodial epidermis are competent to produce morphologically complete setae in gameteless specimens, although both the cell and its product may be smaller than normal.

3. In females with oöcytes greater than about 50 μ in diameter, a detectable group of chaetogenic cells is often present within three days of decapitation; this tissue does not normally appear until the oöcytes are 95 μ in diameter.

4. Chaetogenesis already in progress may be accelerated by decapitation of animals during stage I and early stage II, when the oöcytes are under 140 μ in diameter.

5. Decapitation of animals with oöcytes greater than 140 μ in diameter did not accelerate chaetogenesis.

6. The duration of each setal sac stage has been calculated from the known oöcyte growth rate. Metamorphosis is estimated to occupy about 67 days.

7. The relationship between the setal sac stages and the stages already in the literature is examined.

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LOSS OF ZN^{65} FROM THE CALIFORNIA SEA-MUSSEL MYTILUS CALIFORNIANUS

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Mussels growing on rocks and pilings are useful for detecting minute changes in certain radioisotopes in the local environment. They accumulate certain metals such as zinc, cobalt, manganese and silver to concentration levels far above what is present around them. Therefore, evidence of certain radionuclides, especially those emitting gamma rays, often may be obtained quickly and easily by inspecting the tissues of a few *Mytilus* on a gamma spectrometer. This may be possible even when environmental concentrations are so low that direct radioanalysis of sea water concentrates would be extremely difficult and would require very large samples.

For example, considerable information was gained about the coastal distribution of Zn^{65}, Co^{60}, Mn^{54} and Ag^{110m} through inspection of *Mytilus* populations (Folsom *et al.*, 1963; Nagaya and Folsom, 1964; and Folsom and Young, 1965). This information was obtained even before suitable procedures were available for direct assay in sea water; and procedures for some of these that are effective with very small traces in sea water still have not been reported. Trends of Zn^{65} concentrations owing to fallout were followed at Scripps Pier throughout 1963 and 1964 by sampling a few hundred grams of *Mytilus* tissue. This was done at a period when it was found impossible to obtain significant measurements through chemical concentrations of Zn^{65} from 1000-liter samples of sea water.

The distribution of Zn^{65} in the marine biosphere is of some importance because this radioactive isotope arises from neutron-activations during weapon tests and also from experimental and industrial reactors. For example, this nuclide was found prominent in plankton, tuna, and marine birds near Eniwetok a few weeks after the nuclear explosions there in 1956 (Lowman *et al.*, 1957; Lowman, 1960). Also, it has been found to be the most conspicuous artificial gamma-emitter in marine animals off the Oregon Coast (Watson *et al.*, 1963). The relatively high concentrations of Zn^{65} that persist near the mouth of the Columbia River are attributed to the practice of circulating river water through the reactors at Hanford which activates small traces of stable zinc present in the river water; inputs to the Pacific from this source of about 30 curies per day have been reported (Alexander and Rowland, 1966).

Although monitoring concentrations in certain tissues of an organism like *Mytilus* may be very useful to give early and sensitive warning of small environmental changes, the absolute amounts of these changes and the time when they occurred cannot be estimated without some knowledge of how much this organism concentrates the element and also some knowledge as to how fast it responds to external changes. This paper describes some experiments giving evidence of the response behavior of *Mytilus californianus,* maintained in otherwise natural conditions, following changes in Zn^{65} within the range certain populations may experience.

The experiment was designed to follow the tissue concentrations by repeated harvests over a period of one year after the environmental concentration of Zn^{65} was suddenly reduced. These organisms are very hardy; the same species grows near the mouth of the Columbia River and on the pilings at Scripps Pier. It was found possible to transport large numbers of them without apparent shock from rocks in Oregon, and to study their behavior while exposed in several colonies to the cleaner sea water at Scripps Pier. They were suspended in nets near native populations just beyond the surf zone. So great are the differences in concentration of Zn^{65} observed in Oregon *Mytilus* and in those at Scripps, that this experiment may be viewed as reasonably approximating conditions for the classical step-change experiment. These conditions can be provided under natural circumstances. It would be impossible to provide equal controls in an aquarium.

METHODS

Specimens

About 100 kg. of *Mytilus californianus* were collected on 2 November 1963, at Cannon Beach, Oregon, about 25 miles south of the mouth of the Columbia River. They were packed in ice and brought to La Jolla by air and again put into the sea at Scripps Pier within 24 hours. Clusters of 10 or more were removed together, with care taken to avoid shock. Less than 1% of the individuals died during the transportation. About 300 typical individuals, ranging in length from 8 to 16 cm., were collected.

Exposure at Scripps Pier

Scripps Pier is 300 meters long, extending beyond the normal surf zone to a depth of about six meters. The mussels from Oregon were separated into six colonies and put into perforated polyethylene baskets (45 cm. diameter × 30 cm. deep) and suspended in nylon mesh bags by ropes at the end of the pier. These colonies were supported at mean lower low tide level so they would be exposed as frequently as were the native *M. californianus* on the nearby pilings.

To test for possible effects arising from suspension in nets, three colonies of mussels taken from nearby pilings were suspended from the pier in a similar manner.

Harvesting

Samples were removed from the baskets on 21 dates between 2 November 1963 and 3 November 1964, as listed in Table I, for radioanalyses. Generally, 9 to 10 individuals, ranging from 8 to 16 cm. in length, were sacrificed for each assay. Harvesting was distributed so that the colonies decreased uniformly.

Samples were harvested about twice each month. On the 223rd day, five samples were taken. The first of these was taken from upper layers of the colony as was the usual practice. However, random sampling procedures were carried out on four other samples. In this instance, all individuals were given temporary

Table I

Zinc-65 biological half-life study of the California sea-mussel Mytilus californianus

Collected at Cannon Beach, Oregon: November 2, 1963;
Transferred to Scripps Institution Pier: November 3, 1963.

Code no.	Sample wet weight (grams)	Sacrificed (Date)	t* (days)	Picocuries/wet kg.	
				Zn^{65}**	K^{40}
28	389	11/02/63	0	21,000 ± 210	1590 ± 30
29	390	11/02/63	0	14,400 ± 190	960 ± 40
30	411	11/02/63	0	20,200 ± 200	1200 ± 40
31	397	11/02/63	0	22,100 ± 220	1490 ± 30
32	356	11/02/63	0	25,400 ± 250	1350 ± 30
80	252	11/06/63	3	17,500 ± 180	1590 ± 30
81	321	11/09/63	6	16,400 ± 160	1530 ± 30
82	225	11/12/63	9	20,300 ± 200	1550 ± 50
83	271	11/22/63	19	15,500 ± 160	1730 ± 40
84	410	11/22/63	19	***346 ± 10	210 ± 30
85	295	12/06/63	33	15,200 ± 150	1660 ± 30
86	370	12/20/63	47	12,400 ± 120	1580 ± 30
87	330	1/03/64	61	10,900 ± 110	1370 ± 30
88	297	1/20/64	78	8,700 ± 90	1500 ± 30
89	575	1/20/64	78	***343 ± 20	160 ± 20
90	307	1/30/64	88	10,800 ± 110	1490 ± 30
91	442	2/15/64	104	8,500 ± 90	1510 ± 30
92	276	2/29/64	118	7,120 ± 70	1400 ± 30
93	337	3/14/64	132	5,230 ± 50	1640 ± 30
94	385	3/30/64	148	4,040 ± 40	1550 ± 20
95	610	3/30/64	148	***297 ± 20	220 ± 30
96	341	4/10/64	159	4,000 ± 40	1710 ± 30
97	486	4/24/64	173	2,010 ± 20	1950 ± 20
98	270	5/24/64	203	1,600 ± 20	2140 ± 20
99	430	6/13/64	223	2,870 ± 30	2050 ± 20
100	525	6/13/64	223	2,110 ± 20	2390 ± 50
101	655	6/13/64	223	3,330 ± 30	2120 + 40
102	632	6/13/64	223	2,720 ± 30	2140 ± 40
103	640	6/13/64	223	2,990 ± 30	1950 ± 40
104	500	6/13/64	223	***152 ± 10	160 ± 30
105	424	8/13/64	284	1,250 ± 10	1860 ± 40
106	375	8/13/64	284	940 ± 20	1810 ± 40
107	505	9/13/64	315	840 ± 20	1670 ± 30
108	577	9/13/64	315	930 ± 20	1800 ± 40
109	650	9/13/64	315	*** 84 ± 10	—
110	234	10/13/64	345	790 ± 40	1550 ± 50
111	243	10/13/64	345	1,210 ± 20	1750 ± 40
112	650	10/13/64	345	*** 90 ± 10	200 ± 20
113	660	11/03/64	366	1,100 ± 20	1880 ± 40
114	661	11/03/64	366	940 ± 10	1810 ± 20

* Number of days Oregon specimens were maintained in La Jolla surf before being sacrificed.

** Zinc-65 values were corrected for radioactive decay to November 3, 1963 using a radioactive half-life of 244 days.

*** Cleaned shells of 3–6 mussels.

numbers and four sets of 10 individuals were selected randomly from a total of 150. These randomly selected samples are indicated by crosses in Figure 1. The remaining mussels were divided equally in three baskets and returned to the sea.

Entire soft tissues were cut from the shells, avoiding damage to the organs. These tissues, generally weighing about 300–500 grams, were drained about five minutes on a sieve and then put into a cylindrical container of a type standardized for gamma analysis of wet tissues.

Also, on six occasions during the year, three to six shells were scraped free of soft tissue fragments, barnacles, and algae and were set aside for radioanalyses.

Only occasionally was an empty shell encountered; apparently more than 90% of the Oregon individuals survived until harvested.

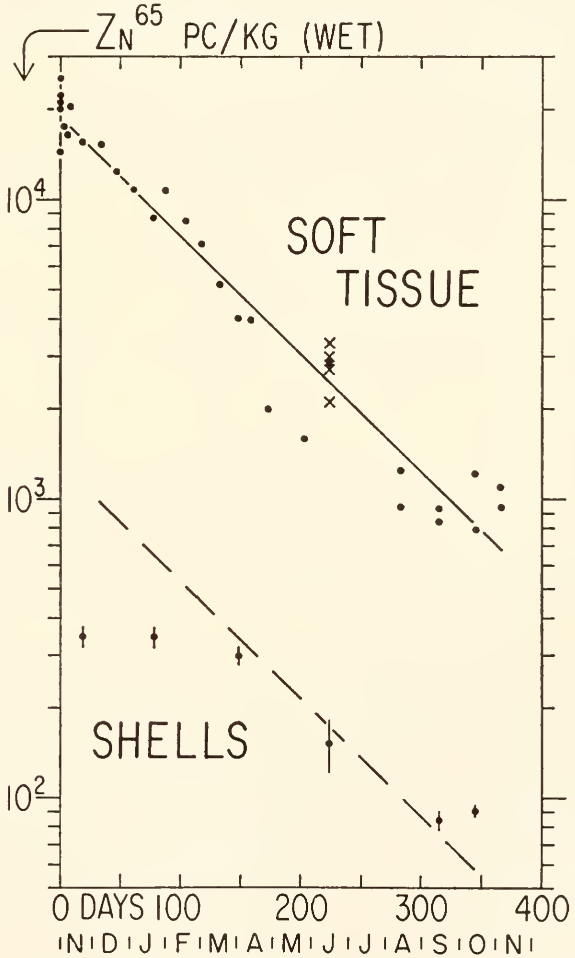

FIGURE 1. Decrease in Zn^{65} concentration in *Mytilus californianus* after translocation from Cannon Beach, Oregon, to La Jolla, California. Correction has been made for radioactive decay. Crosses indicate randomly selected samples.

Gamma analysis

Concentrations of Zn^{65} and K^{40} were determined in the wet tissues and shells without chemical processing by use of a 256-channel gamma ray spectrometer. The specimens, in standardized (10 cm. diameter) plastic containers, were placed on top of a shielded NaI detector (5″ × 4″) and a count was made for 16 hours or more. Corrections were made for sample geometry and background spectra were subtracted digitally.

RESULTS

Observed activities

Concentrations of Zn^{65} and K^{40} and their standard deviations of counting are given on a wet basis in Table I. All Zn^{65} activities were corrected to 3 November 1963 assuming a radioactive half-life of 244 days.

Distribution of Zn^{65} in Mytilus tissues

Analyses of the separate organs of the Oregon specimens were not made. However, Table II summarizes the distribution of Zn^{65} obtained by dissecting individuals of the same species that were collected in 1963 from the rocks of Bodega Head, California, and which had been influenced by fallout Zn^{65} for more than one year. Each animal was dissected into the portions listed, and because of the differences in organ sizes, varying numbers of animals were used to make up the several tissue samples.

TABLE II

*Distribution of Zn^{65} in Mytilus tissues**

Tissue	Number of mussels	Wet weight/ individual (grams)	% of whole weight	% of Zn^{65} in whole
Undissected whole	6	190	—	—
Kidney	30	2	1.1	50
Digestive glands	30	7	3.7	19
Gonads	30	13	7.0	8
Ctenidia	30	7	3.7	5
Musculature	18	28	15.0	14
Shell	3	130	69.5	4

* Averages of *Mytilus californianus* 16 cm. in length from Bodega Head, California, collected in 1963.

It will be seen that the shell contained only 4% of the Zn^{65} but contributed about 70% of the entire weight. Half of the Zn^{65} was found in the kidney tissues that constituted little more than 1% of the weight.

Observed decreases in the Zn^{65} concentrations

The trend of concentrations of Zn^{65} (corrected for radioactive decay) in the soft tissues of the transplanted Oregon mussels, as seen in the semi-logarithmic plot in Figure 1, was fitted with a straight line by the method of least squares.

The response was assumed to be a simple exponential function $A_t = A_0 e^{-bt}$, where A_0 is the initial concentration of Zn^{65}, A_t is the concentration at any later time t, and b is a constant related to the loss rate of Zn^{65}. The time required for the concentration to decrease to half is called the half-time or half-life. After correction has been made for radioactive decay, as in this case, the concentration decrease is due to biological processes alone, and the half-time is called the biological half-life, T_b. From the equation above, it is seen that $T_b = \log_e 2/b$, where b is the slope of the best-fit line in Figure 1. The slope of this line corresponds to a biological half-life of 76 days with standard deviations of ±3.5 days providing each measurement is given equal weight. Based alternatively upon K^{40} content, the Zn^{65} attenuation fitted essentially the same rate over the year, having a half-life of 75 ± 3.3 days.

In addition to the concentration decrease due to biological processes, Zn^{65} is lost due to radioactive decay, decreasing exponentially at the rate of half per 244 days. The equation governing this process is the same as that assumed for the biological turnover, $A_t = A_0 e^{-rt}$, where r is the radioactive decay constant and is related to the radioactive half-life T_r: $r = \log_e 2/T_r$. The biological and radioactive decay constants are additive, and together yield a loss rate called the effective half-life, which describes the actual rate at which the Zn^{65} concentration would decrease in these mussels growing in an environment free of this radionuclide. In our case, combination of biological and radioactive half-lives of 76 and 244 days, respectively, gives an effective half-life of 58 ± 2.7 days for Zn^{65} in the soft tissues.

The concentration in the shells was slower and apparently variable. In Figure 1, a dashed line corresponding to a 76-day biological half-life has been added to emphasize that the concentration in shells decreased slower than this rate during the first 100 days and possibly during the last weeks of the test.

Scatter in measurements

Scattering of measurements in soft tissues was much greater than attributable to the counting fluctuation. Standard deviation of departure of measurements from the fitted line was ±25%. Standard deviation of the mean concentrations in the five initial samples ($t = 0$) was ±19%, and it was ±16% for the entire five samples collected on day 223. For the four samples collected on day 223 by sampling individual mussels at random, the standard deviation of the mean values was ±20%.

Controls

Suspending mussels artificially in baskets apparently had no significant effect on their Zn^{65} or natural K^{40} content. Table III supports this by comparing concentrations in samples of local *M. californianus* that were transplanted from Scripps Pier pilings into suspended baskets with concentrations observed in samples from the natural colonies on the nearby pilings.

Potassium concentration in soft tissues of the local control organisms, 1790 ± 100 pc./kg., was essentially the same as the value (1680 ± 290 pc./kg.) observed in the Oregon mussels harvested during the year. Here, means and standard deviations of separate measurements are compared; in Table III the estimated errors arising from counting K^{40} radioactivity are listed separately.

TABLE III

Control test of influence on local mussels of suspension in net bags

Date sampled	Sampled from local population suspended in net bag			Sampled from nearby natural population on piling		
	Wet weight (grams)	Picocuries/wet kg.		Wet weight (grams)	Picocuries/wet kg.	
		Zn^{65}	K^{40}		Zn^{65}	K^{40}
11/12/1963	286	47 ± 7	1800 ± 40	—	—	—
11/14/1963	—	—	—	279	42 ± 6	2100 ± 40
11/22/1963	145	51 ± 12	1900 ± 60	404	47 ± 6	2000 ± 20
12/20/1963	—	—	—	366	36 ± 6	2150 ± 20
12/26/1963	135	36 ± 4	1850 ± 40	—	—	—
1/03/1964	346	30 ± 5	1600 ± 20	186	19 ± 9	1650 ± 30
1/28/1964	—	—	—	527	23 ± 7	1800 ± 40
1/30/1964	270	23 ± 5	1800 ± 20	—	—	—
2/14/1964	270	45 ± 6	1850 ± 20	530	32 ± 7	1950 ± 40
2/14/1964	—	—	—	531	30 ± 6	2050 ± 20
2/14/1964	—	—	—	546	15 ± 6	2000 ± 20
2/14/1964	—	—	—	410	46 ± 6	1700 ± 30
2/14/1964	—	—	—	362	35 ± 6	1650 ± 30
3/04/1964	—	—	—	448	20 ± 7	1750 ± 40
3/25/1964	375	17 ± 6	1700 ± 30	—	—	—
Averages and Standard Deviations	261 ± 92	36 ± 13	1790 ± 100	415 ± 113	31 ± 11	1890 ± 190

DISCUSSION

The species *M. californianus* is much more abundant in intertidal regions where there is surf than in quiet harbors. It would be difficult to provide conditions in an aquarium simulating the environment of these organisms. In this experiment, test organisms were moved as gently as possible to an environment which closely resembled the natural one.

Surf temperatures at La Jolla average over one year about 5° C. higher than reported for the Oregon coastal waters. Oregon coastal seaboard temperatures generally range between 9° and 15° while at Scripps Pier the range is 14° to 22° C. However, this intertidal organism is known to be tolerant of relatively large temperature differences. For instance, Bullock (1955) found similar pumping rates for *M. californianus* from 6.5° C. water (48° N) and from 12° C. water (34° N).

These mussels tolerate some salinity change also. Salinities along the coast near the Columbia River fluctuate, sometimes falling below 26 parts per thousand (Park *et al.*, 1965). At other times, the river's outflow becomes displaced so that ocean water having normal salinity may reach the coast; during periods of upwelling, even higher salinities occur. The salinity at La Jolla, about 33.6 parts per thousand, falls within the range experienced by the Cannon Beach mussels.

The trend of measurements themselves shows no clear sign of shock following transplantation. For example, the intercept of the fitted straight line (18,900 ± 1300 pc./kg.) falls near the mean concentration (20,600 ± 400 pc./kg.) actually measured in these specimens harvested initially at Cannon Beach. Moreover, most of the later measurements do not range more widely from the fitted trend than do the five initial samples around their mean.

In view of the concentration differences found in the separate organs listed in Table II, it is not realistic to attribute attenuation of traces of zinc to a single process. Nevertheless, the slope of the simple exponential curve does predict approximately how fast, exclusive of radioactive decay, the concentration of Zn^{65} in the soft parts of *M. californianus* about 11 cm. in length will decrease when this nuclide is removed from its environment. The approximation presumably is more valid over a full year's period, and for temperature and salinity conditions similar to those found in southern California waters.

The purpose of this experiment was to determine the rate at which zinc was lost from mussels of a convenient size range growing in a natural environment. However, in order to make these data applicable to *Mytilus* of different sizes, an attempt will now be made to estimate the biological turnover rate independent of growth. The average length of our specimens sampled in the first few months of the experiment was 11 cm., and during the last few months it was 12 cm. This increase is consistent with the data of Coe and Fox (1942), who found that the 11-cm. mussels grew about 1 cm. in length in one year. Moreover, Fox and Coe (1943) reported a logarithmic relationship between tissue weight and length of shell for a mixed population of *M. californianus* sampled at Scripps Institution Pier and nearby rocks. From this relationship, we have estimated that our average-sized specimens increased their tissue weight (in the waters of La Jolla) by 25% during the year. Therefore, it can be shown that, if changes in concentrations owing to weight increases are removed (as well as the effect of radioactive decay), the loss rate is described by a half-life of 82 rather than 76 days.

Unfortunately, the very high concentrations localized in the kidneys (see Table II) may have caused inhomogeneities in the samples of soft tissues sufficient to contribute part of the observed scatter in the measurement that should not be attributed to the sampling variance. This sort of geometric variation could not occur during analyses of shells because they were broken into small pieces and were well mixed.

Dissection and separate analyses of the several soft tissues were not attempted in this series of tests, but occasional analyses of shells disclosed an interesting difference in response characteristic. Six analyses were made on the shells, which have some special merit because of the relatively large weight of these organs. It is not surprising that the overall loss of Zn^{65} over the whole year was slower from shells than from soft tissues. On the other hand, the observed initial lag is puzzling. Less than 15% loss was measured during the first five months; thereafter the rate increased.

If Zn^{65} had been deposited in the shells more or less uniformly throughout their thickness during growth in Oregon, the losses at La Jolla should have become slower as time went on and as surface layers became depleted. The attenuation curve under this hypothesis would be expected to exhibit its steepest slope initially and lesser slopes thereafter. This is certainly not what is seen in the shell purging measurements in Figure 1, although there is some suggestion that slopes may be decreasing at the end of the experiment.

Seasonal changes in the growth rate may provide a partial explanation. It will be noted in Figure 1 that the loss rate for the shells was accelerated beginning in the spring. There is some indication that this may also be the case for the soft tissues, although more data would be needed to resolve this point. It has been shown (Coe and Fox, 1944) that growth of this species at Scripps Pier increases at this time, and presumably so would the rate of adjustment to the environment.

We are grateful for the counsel of Professor Milner B. Schaefer, Director of the Institute of Marine Resources of this University. We also thank Professor Charles Osterberg and the Department of Oceanography, Oregon State University, for assistance in our collecting in Oregon and for providing temperature and salinity data. This work was supported by the U. S. Atomic Energy Commission and used analytical facilities developed with the aid of the Office of Naval Research of the U. S. Navy.

SUMMARY

To determine the loss rate of Zn^{65} in the California sea-mussel, colonies of *Mytilus californianus* averaging 11 cm. in length were translocated from the vicinity of the Columbia River mouth to southern California waters, where much lower levels of Zn^{65} occur. The colonies were suspended in nets at Scripps Institution of Oceanography Pier, and were sampled periodically for one year. During this time, the concentration of Zn^{65} in the soft tissues (after deducting radioactive decay) decreased by more than 97%. This decrease appears to be described satisfactorily by a single exponential function, with a biological half-time for growing organisms of 76 ± 3.5 days. If radioactive decay is not excluded, the observed loss rate of Zn^{65} is described by an "effective half-life" of 58 ± 2.7 days.

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ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY 1967

ABSTRACTS OF SEMINAR PAPERS

July 11, 1967

Is there a physiological limit to the response of melanophores in Uca to the melanin-dispersing hormone? Milton Fingerman, K. Ranga Rao and Clelmer K. Bartell.

The melanophores of the fiddler crab, *Uca pugilator,* appeared to be capable of an increasing response with increasing concentration of melanin-dispersing hormone to a particular level of overall response only, and any additional increase in hormone concentration evoked no further response (Sandeen, *Physiol. Zoöl.,* 23: 337, 1950). Sandeen then suggested that there is a physiological limit to this response whereby very high concentrations of the hormone could not be assayed directly. Her extracts were prepared by triturating the tissues directly in sea water. We, however, found that a hormone preparation obtained by extracting eyestalks of *Uca pugilator* with absolute ethyl alcohol evoked with higher concentrations a larger response than that obtained when eyestalks were extracted directly in physiological saline or distilled water. The melanin-dispersing response evoked by the alcohol extract increased sharply with increasing concentration between 1 and 80 eyestalks per ml. In contrast, dosage-response curves for aqueous extracts did not rise as sharply and were nearly flat in the range of 1 to 10 eyestalks per ml., where Sandeen worked. At the highest concentration used, the alcohol-soluble extract caused more than three times the response evoked by aqueous extracts. Our results with aqueous extracts have essentially the same magnitude of response reported by Sandeen. However, the observations with alcohol extracts do not support the hypothesis that there is a physiological limit to the response of these melanophores to melanin-dispersing hormone even with extracts at least eight times as concentrated as those used by Sandeen.

Supported by Grant GB-5236 from the NSF.

Hormones controlling the white chromatophores of the fiddler crab, Uca pugilator. K. Rango Rao, Milton Fingerman and Clelmer K. Bartell.

Investigation of the white chromatophores in *Uca pugilator* from Woods Hole revealed the existence of a white pigment-concentrating hormone only. The white pigment of eyestalkless specimens was maximally dispersed. In uninjected Woods Hole *Uca pugilator* this pigment would not concentrate maximally (Brown and Sandeen, *Physiol. Zoöl.,* 21: 361, 1948; Sandeen, *Physiol. Zoöl.,* 23: 337, 1950). Consequently, assay for a white pigment-dispersing hormone would be quite difficult with these crabs. However, examination of *Uca pugilator* from Panacea, Florida, revealed that the white pigment of intact individuals became maximally concentrated when crabs were placed on a black background. The combination of eyestalkless crabs and intact crabs on a black background would allow assay for white pigment-concentrating and white pigment-dispersing hormones, respectively. Experiments were, therefore, designed to determine whether or not *Uca pugilator* possesses both hormones. Extracts of the optic ganglia, sinus glands, supraesophageal ganglia, and thoracic ganglia prepared directly in physiological saline evoked only dispersion of the white pigment. However, acetone extraction of the same tissues yielded an acetone-soluble fraction that caused concentration of the white pigment while the acetone-insoluble fraction contained the white pigment-dispersing hormone. The circumesophageal connectives, in contrast, contained only the white pigment-concentrating hormone. The white pigment-concentrating and white pigment-dispersing hormones appeared to be mutually antagonistic. It is highly unlikely, furthermore, that melanin dispersion and white pigment dispersion are caused by a single hormone.

Supported by Grant GB-5236 from the NSF.

Heterologous reactions among aminoacyl t-RNA ligases. ROBERT B. LOFTFIELD AND E. ANN EIGNER.

An enzyme (the aminoacyl t-RNA ligase) catalyzes both the reaction of a particular amino acid with ATP to form an enzyme-bound activated amino acid and the subsequent reaction of this complex with the amino acid specific t-RNA to form 2′ aminoacyl t-RNA. All evidence indicates that every species possesses a distinct enzyme and one or more t-RNA molecules for each of the naturally occurring 20 amino acids.

Just as similar proteins possess different structures in different species, the t-RNA's of different species appear to vary substantially in composition and sequence. However, all the t-RNA's examined have a 5′ guanosine group and a 3′ cytosine cytosine adenine group. Moreover, studies of the binding of aminoacyl t-RNA's to ribosomes (the presumed next step in protein biosynthesis), indicate that t-RNA's specific to a particular amino acid have some sequence, a di- or tri-nucleotide, in common regardless of the tissue of origin.

The purpose of the present investigation was to determine whether the ligases "recognize" the same part of the t-RNA as is used in the genetic code. Put into other words, is the "trinucleotide anti-codon" a significant factor in the binding of a specific aminoacyl t-RNA to the ligase regardless of whether the pairs are derived from a single species?

We find that among toadfish, starfish, *E. coli* and yeast the leucine-specific and the valine-specific t-RNA's are aminoacylated by the corresponding heterologous enzymes although the optimal rates vary from 1/500 of the homologous reaction to several times that of the homologous reaction. Thus it was noted that *E. coli* enzymes react, in general, extremely slowly ($<2\%$) with the t-RNA's of yeast starfish or toadfish, whereas the yeast enzymes actually react better with toadfish t-RNA than with yeast t-RNA.

All assays were established as initial rates (as opposed to extents of reaction in a particular time interval). No heterologously aminoacylated t-RNA accepted more (^{14}C)-amino acid than it accepted from the homologous enzyme and all preliminary evidence indicates that the heterologously labeled t-RNA's are indistinguishable from the natural product. Hence it appears that the structure of the t-RNA and its conformation may exert a 1000-fold effect on the actual rate of the reaction while the necessary condition for t-RNA-enzyme recognition resides with the "semi-universal" anti-codon of the t-RNA.

JULY 18, 1967

Behavioral responses of isolated tentacles of Hydra. NORMAN B. RUSHFORTH.

In external recordings made at the base of an intact hydra small potentials (*ca.* 50 μv.) are observed which are correlated with the contractions of individual tentacles. Such pulses (TCP's) originate from the tentacles and presumably spread passively down the body column. They may be recorded externally from the base of an excised tentacle as large compound potentials (up to 10 mv. and of 200 msec. duration).

In *H. pirardi* and *H. pseudoligactis* isolated tentacles spontaneously produce TCP's in bursts of three to five pulses. The inter-burst interval is much greater in *H. pseudoligactis* (209 ± 26 sec.) than *H. pirardi* (44 ± 5) while the mean interval per burst is much shorter: 1.9 ± 0.4 compared with 3.7 ± 0.3, respectively. Excised parts of tentacles give TCP bursts similar in temporal patterning to those of the whole tentacle.

Photic, mechanical and chemical stimuli applied to isolated tentacles affect the occurrence of endogenous contractions and TCP's. The frequency of TCP bursts is increased in both species in periods of strong light. The number of pulses per burst is increased and the inter-burst interval is decreased during illumination. Single TCP's may be induced in *H. pseudoligactis* by short pulses of mechanical agitation. The tentacle rapidly adapts to such stimulation. A change in the pattern of spontaneous activity is also effected when the isolated tentacle catches a single *Artemia* or is exposed to $10^{-5}M$ reduced glutathione. The normal bursts of contractions are inhibited and the tentacle gives asymmetric bending movements followed by uncoordinated writhing. Such movements are characteristic of the tentacles of the intact animal during feeding. Associated with such writhing are frequent single monophasic potentials (up to 5 mv. and up to 200 msec. duration).

These results indicate that the pacemaker activity of an isolated hydra tentacle is modified by external stimulation frequently in a manner similar to that observed when the tentacle is attached to the animal.

Supported by grants from the National Institutes of Health.

Behavioral aspects of Histriobdella homari, an annelid commensal of the American Lobster. JOSEPH L. SIMON.

Specimens of the commensal polychaete, *Histriobdella homari,* were found inhabiting the branchial chambers of all 27 American lobsters (*Homarus americanus*) collected during September and December, 1965, and between May and August, 1966, in Great Harbor, Woods Hole. The number of commensals varied between 3 and 648 per lobster. There were no apparent correlations between number of commensals and size or sex of lobster. Data indicated the possibility of seasonality in occurrence of commensals. Laboratory experiments showed clearly that *Histriobdella* can migrate from a confined volume of sea water to the lobster branchial chamber and from one lobster to another.

Supported in part by a grant from the Ford Foundation to the Marine Biological Laboratory, Systematics-Ecology Program.

JULY 25, 1967

Alteration of smooth muscle receptors by heat and urea. SEYMOUR EHRENPREIS AND JEROME H. FLEISCH.

Rat stomach fundal strips (Vane preparation) in Krebs-bicarbonate buffer at 37.5° C. were subjected to heat and urea treatments in an attempt to obtain information about the molecular nature of receptors for various biogenic amines. Optimum conditions for achieving differential effects were 47° C. for 20 minutes (the heat treatment) and $2M$ urea (highly purified, Mann Laboratories) for 19 minutes (the urea treatment). Following treatment, the tissues were washed repeatedly to equilibrate under initial conditions and then tested with the various agonists. These treatments caused no alteration in the dose-response curve to KCl, a non-specific smooth muscle stimulant. The heat treatment produced no effect on responses to acetylcholine or bradykinin. A marked decrease in slope of the serotonin dose-response curve was noted although the maximum contraction height could be achieved. This required a 1000-fold higher concentration than the control. Slopes of dose-response curves to vasopressin and angiotensin were greatly depressed with only 50% of the maximum contraction height attained at the highest concentrations used (more than 1000 times the control). Urea treatment produced the following effects: Decrease in slopes of dose-response curves to bradykinin, acetylcholine, and serotonin, with maximum contraction heights achieved at 19, 375, and 5,000 times control concentrations, respectively. The effect of urea on the response to angiotensin was the same as with heat. These various results are interpreted as follows: Receptors for all agonists examined are proteins, the acetylcholine and bradykinin receptors being relatively heat-stable but partially denatured by urea. The serotonin receptor is partially denatured by both heat and urea, while receptors for vasopressin and angiotensin are far more labile, heat or urea causing total destruction of a fraction of these receptors.

Supported in part by USPHS grants HE 08597, NB 7050, GRS grant FR 95360-06 and NSF grant GB 5654.

The relationship between soluble and insoluble protein in the lens. SEYMOUR ZIGMAN AND SIDNEY LERMAN.

Studies of the insoluble or "albuminoid" protein of young rat lenses have indicated that it is an insoluble product of the lens protein gamma (γ)-crystallin formed in part by the conversion of —SH to —SS— bonds. This conclusion is based upon observations of the tendency of the three soluble lens proteins to become insoluble under mild conditions, and direct comparisons of composition and physicochemical properties of solubilized albuminoid and these proteins.

Only γ-crystallin (—SH content ≅ 150 μM/gm.) became insoluble when dilute solutions of lens soluble proteins purified by DEAE-cellulose column chromatography were kept at room temperature in air, or were incubated at 37° C. in 95% O_2:5%CO_2. This insolubilization was temperature-dependent, was inhibited by mercaptoethanol and other —SH reagents, and was enhanced by oxidizing agents, such as $K_3Fe(CN)_6$ and H_2O_2. Other lens proteins (*i.e.*, α-crystallin) and high levels of urea (8 M) also inhibited γ-crystallin insolubility, but the process continued even in the absence of O_2. It seems likely that other protein and amino acid interactions are also involved.

Reductive sulfonation of the albuminoid using Na_2SO_3 and $Na_2S_4O_6$ at pH 9.9 in 8 M urea led to a completely water-soluble —SH-free material which was found to be nearly identical with γ-crystallin in its ultraviolet extinction ($E_{1}^{\%1\,cm} = 17$–18), sedimentation rate (2*S*), amino acid composition (high arginine, glycine, aspartic and glutamic acids, and tyrosine) and electrophoretic mobility on polyvinyl chloride thin layers (with 2 components moving 9 and 12 cm./hr.). It would thus appear that intact —SS— bonds are important in maintaining the insoluble state of this protein.

Dogfish γ-crystallin and albuminoid are also similar by the above criteria, but bovine α-crystallin strongly resembles albuminoid.

Supported by NIH grant #NB 03081 and the Rochester Eye Bank.

Demonstration of phospholipid splitting as factor responsible for venom action on squid giant axon. PHILIP ROSENBERG AND ELEONORA CONDREA.

Certain snake venoms, such as cottonmouth moccasin or ringhals, will in relatively low concentrations (15 µg./ml.) render the squid giant axon sensitive to curare, acetylcholine and other compounds which normally do not affect conduction. The venoms also markedly increase the permeability of the axon to any poorly penetrating compound (*Ex.* acetylcholine, curare, sugars, amines etc.) and disrupt the Schwann cell. Higher concentrations of these venoms (50 µg./ml.) will themselves irreversibly block conduction.

Phospholipase A isolated from ringhals venom mimicked in all respects the action of the whole venom. Both venom and phospholipase A cause above described effects if the giant axon used has adhering small nerve fibers, but they are inert on the giant axon if free of all adhering nerves. Two-dimensional thin layer chromatographic analysis of the axonal phospholipids indicated that both venom and phospholipase A (50 µg./ml.) caused up to 50% splitting of the individual axonal phospholipids (lecithin, phosphatidyl ethanolamine and phosphatidyl serine). Lower concentrations (15 µg./ml.) split up to 20% of these phospholipids. The hydrolysis of phospholipids in whole nerve and in separated envelopes after extrusion of axoplasm was about equal in preparations of giant axons free of adhering nerve fibers and in those containing them. These findings indicate that phospholipid splitting *per se* is not responsible for venom or phospholipase A action. Lysophosphatides, produced as a result of phospholipid hydrolysis by phospholipase A, have detergent actions and may be responsible for venom effects. They would be produced in greater amounts in the axons containing adhering fibers. Indeed lysolecithin causes block of conduction in both preparations. In addition, phospholipase C (20 mg./ml.) whose action does not give rise to lysophosphatides has little or no effect on axonal conduction or permeability even though splitting more of the phospholipids than phospholipase A.

AUGUST 1, 1967

The origin of fast photoelectric effects in the squid retina. ROBERT E. MCGAUGHY AND WILLIAM A. HAGINS.

When a dark-adapted squid retina is illuminated with an intense flash of light, a transretinal voltage develops which arises from two different processes. The larger delayed component, a conventional electrophysiological receptor voltage, is abolished by glutaraldehyde fixation while the smaller component (the "fast photovoltage" or FPV) persists after fixation. The FPV waveform is characteristic of the retina's visual pigment composition.

This work reports that the FPV arises from currents flowing across the photoreceptor cell membrane in the region of light absorption. These currents then spread along the cells as if the photoreceptors were electrical cables. The evidence for this conclusion comes from experiments on slices of retina placed on a microscope stage so that the photoreceptors can be seen from the side. The slice is illuminated with a 30-micron slit of light perpendicular to the long axes of the cells, and the voltage difference is measured between two electrodes placed on the outer segments at different distances from their ends. The FPV response reverses sign when the slit is moved from one electrode to the other and, although it gets smaller as the slit is moved further from the electrodes, there are still detectable responses as far away as 100 microns from them. This shows that currents originate in the region of the stimulus and spread to distant regions of the outer segments. However, if the FPV were produced by a summation of longitudinal voltage generators spaced all along the photoreceptors, such a voltage reversal would not occur and no response would be observed from slit stimuli outside the region between the two electrodes.

It is believed that the local currents are caused by ions migrating in the retinal impedances in order to neutralize dipole moments produced across the cell membrane when the visual pigment is illuminated.

AUGUST 8, 1967

Interstitial marine gastrotrichs from Woods Hole, Massachusetts. WILLIAM D. HUMMON.

Seven previously described species have thus far been collected during a study of the marine Gastrotricha of Cape Cod: *Aspidiophorus marinus* Remane, 1926; *Chaetonotus aculifer* Gerlach, 1953; *Xenotrichula beauchampi* Levi, 1950; *Pseudostomella roscovita* Swedmark, 1956; *Tetranchyroderma papii* Gerlach, 1953; *Turbanella cirrata* Papi, 1957; and *Turbanella cornuta* Remane, 1925. All but *Aspidiophorus marinus* have been reported from the Mediterranean or Adriatic Coasts, and all but *Tetranchyroderma papii* and *Turbanella cirrata* from the European Atlantic Coast. Several have been recorded from other localities of the world as well. Only *Turbanella cornuta* has previously been reported from North America, from Puget Sound and the San Juan Archipelago, Washington (Wieser, 1957; Hummon, 1966).

Three Woods Hole beaches have been studied at low tide, Nobska Beach (facing Vineyard Sound) and Crane's and MBL Beaches (facing Buzzards Bay). The beach sand at these localities is medium to coarse, stratified, and poorly sorted. Nobska Beach, more variable than the others, shows greater stratification and better sorting.

Chaetonotus aculifer is the only abundant chaetonotoid, inhabiting the shallow sand at high-, medium-, and low-tide elevations. *Turbanella cornuta* occurs only on Nobska Beach, at moderately shallow depths in the high-tide region. *Turbanella cirrata* inhabits 20–60-cm. depths at high- and 5–20-cm. depths at mid-tide elevations. *Pseudostomella roscovita* inhabits only 10–40-cm. depths at the high-tide elevations of Nobska and MBL Beaches. If *Tetranchyroderma papii* occurs with *Pseudostomella* at these locations, it inhabits only the 50–60-cm depths. However, on Crane's Beach, where *Pseudostomella* has not been found, *Tetranchyroderma* occupies depths of 5–60-cm. at high-tide elevations. It also inhabits 5–30-cm. depths at mid- and 0–15 cm. depths at low-tide elevations on all three beaches.

Contribution number 129 from the Systematics-Ecology Program, Marine Biological Laboratory. Supported by an NDEA Fellowship, University of Massachusetts, Amherst.

Ecological studies of New England nudibranchs. BARRY A. WADE.

Nudibranchs of the Cape Cod region have been studied over a period of two years. Life histories of the more common aeolids have been investigated by field and laboratory observations in order to explain their sporadic occurrences in the area. In addition, the effect of Cape Cod as a southern boundary for the boreo-arctic species has been examined.

Twenty-two species have been collected comprising 13 aeolids, 2 dendronotids, and 7 dorids.

Growth of the majority of species is more rapid and the life spans shorter at Cape Cod than in the eastern Atlantic. However, growth rates may vary with the source of food and life spans shortened because of its lack. The shortest normal life span is 3 to 4 weeks in *Tergipes tergipes.* When there is a food shortage, negative growth may occur with the development of "air" bubbles in the liver system of aeolids.

Of the hydroid-eating species, only one (*Favorinus pilatus*) occurs in greatest abundance in the summer. Most of the others have spring and fall maxima. However, their populations on the north side of Cape Cod occur from 1 to 3 months earlier than those on the south side, and there is also a time lag in their reproductive periods. This phasing of populations cannot be closely correlated with the temperature differences of the water masses although they are obviously important. Food is also a factor although its precise effect is unclear. It is believed that the populations at Cape Cod are recruited by settlement of larvae from more northerly populations and that the most successful species are those with short life spans and long breeding seasons. The majority of species are at or near the southern end of their range at Cape Cod.

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AUGUST 15, 1967

Electrically induced spawning in the male and female horseshoe crab, Limulus polyphemus. W. W. SCHRANK, R. L. SHOGER, L. M. SCHECHTMAN AND D. W. BISHOP.

Spawning can be readily induced in mature animals by external stimulation with the standard "MBL *Arbacia* stimulator" (11 v., 60 cps.). Sperm so obtained are viable, can be activated to intense motility and/or the acrosome reaction, and can fertilize electrically spawned eggs. Shedding of gametes is uni- or bilateral, depending on electrode positioning relative to the genital openings on the operculum. Spawning is rapidly induced (< 0.5 sec.) and ceases when the electrodes are removed. If the electrodes are left in place until egg extrusion terminates, 2000 to 3000 eggs can be collected; re-stimulation of the same female within 15 minutes again induces spawning of many eggs. During this interval, mature eggs presumably move down from under the carapace into the main trunk of the oviduct.

Unlike *Arbacia,* in which electrical stimulation causes ovarian muscular contraction, spawning in *Limulus* is mediated through a neuromuscular mechanism which effects a longitudinal muscular contraction of the oviduct and the opening of the genital pore. Motor innervation of the oviduct is provided through a nerve (n.n. 8) which originates in the so-called accessory brain, a part of the fused ganglia of the circumesophageal collar. Distally in the region of the oviduct, this nerve trunk is joined by a small, lateral muscle bundle which originates on the abdominal endochondrite of the opercular segment and inserts on, or in the region of, the oviduct. Stimulation of either nerve or muscle by means of a Grass, model SD-5, square-wave stimulator induces unilateral spawning; ventral cord stimulation causes bilateral oviduct contraction and shedding. After nerve section, shedding is induced by stimulation of the distal stump with a 0.5 msec. stimulus of 2 v. at 60 cps. or 4 v. at 6 cps. Stimulation of the proximal root fails to elicit an oviduct response on either the sectioned or contralateral side.

This study was made in the Fertilization and Gamete Physiology Training Program (NIH grant T1 HD-26-06).

The development of oocytes in the brine shrimp, Artemia. JOHN H. LOCHHEAD AND MARGARET S. LOCHHEAD.

In each ovary of *Artemia,* oogonia form a strip along the full length of one side, while special somatic cells occupy a similar strip along the other side. In the area in between, long twisting chains of cells occur, curling over each other in a complex, tangled mass. The existence of these chains can be recognized only in living, teased preparations, and perhaps for this reason they have been overlooked by previous workers. Each chain is attached at one end to the oogonia, where it originates by repeated mitoses. Within each chain, one particular cell, nearest to the special somatic cells, starts to increase in size and soon grows into a large yolky oocyte. The remaining cells of the chain become polyploid nutritive cells, each with up to a dozen nucleoli and a notably large amount of cytoplasmic RNA. Staining for protein in these cells is relatively weak.

The number of nutritive cells in a single chain may be about 70, roughly 35 on each side of the oocyte. This number sharply decreases in the last stages of the growth period. From a few especially favorable histological sections, it would seem that at a certain stage the oocyte receives an inflow of cytoplasm from the two nutritive cells to which it is directly attached. The nuclei of these cells degenerate and appear not to enter the oocyte. Presumably, the process repeats after the next two nutritive cells have been drawn into a close attachment with the oocyte.

Other histological evidence indicates that the special somatic cells may also have an important role in the nutrition of the oocytes.

Temperature acclimation in Tetrahymena pyriformis. L. C. STONER AND P. B. DUNHAM.

Physiological responses to reduced temperature have been studied in *Tetrahymena pyriformis* strain W, a fresh-water ciliate. Observations on the contractile vacuole and the ability of the cells to maintain intracellular potassium provided evidence that the cells became acclimated to the low temperature. Acclimation is taken to mean compensatory changes, not readily reversible, in organisms subjected to an environmental stress. All experiments were conducted on cells in the 2% proteose-peptone growth medium; all experimental temperature changes were between 23° C. and 11° C.

The mean rate of extrusion of fluid by the contractile vacuole at 23° C. was 0.8 $\mu\mu$l./minute/cell. After a drop in temperature at a rate of 0.4° C./minute, the rate of fluid extrusion by the contractile vacuole was 0.1 $\mu\mu$l./minute/cell. Forty days later, still at 11° C., the rate of fluid extrusion was 0.5 $\mu\mu$l./minute/cell, indicating that acclimation to the low temperature had taken place.

Tetrahymena maintains a high internal potassium concentration relative to the environment. The effects of various rates of temperature drop were studied on the intracellular potassium (K_i). With the fastest temperature drop, 0.4° C./minute, K_i fell from 23.5×10^{-14} to 11.9×10^{-14} moles/cell in 135 minutes. With a temperature drop of 0.2° C./minute, the extent of K_i loss was the same, but the rate of loss was significantly slower. After both of these temperature drops K_i eventually returned to the original level, but the return was slower after the more rapid temperature drop. At the slowest rate of temperature drop, 0.1° C./minute, no loss of K_i was observed over a period of several days. Under the latter condition it was concluded that the rate of acclimation was greater than the rate of temperature drop. This system provides an opportunity for a detailed study of the cellular events accompanying acclimation to temperature changes.

Supported by NIH grant GM-11441-03.

Analysis of invertebrate distribution in the intertidal zone of Barnstable Harbor. ROGER H. GREEN, KATHARINE D. HOBSON AND STUART L. SANTOS.

A year-round study of the causes of mortality in *Gemma gemma* (Totten) (Pelecypoda, Veneridae) in the intertidal zone of Barnstable Harbor, Massachusetts, was begun in October 1966. A region of about 40,000 m.^{2} (*G. gemma* occurring throughout), ranging from 40 cm. to 100 cm. above mean low water, was chosen for subdivision into 3 areas of maximum faunal difference. Between 8 November 1966 and 16 November 1966, 400 samples of 0.1 m.^{2}, 10 cm. deep, were taken at 10-m. intervals throughout the region. Each sample was sieved through a 1-mm. mesh screen, and the animals retained were later identified. Fifty-four of the total of 67 species occurred in more than one sample. The species were distributed fairly equally among the Mollusca, Crustacea and Polychaeta.

These data were analyzed by an association analysis method of Williams and Lambert (1960) on a C.S.I.R.O. (Canberra) computer. This analysis produced three groups of samples for which the faunal differences among groups were maximized and those within groups minimized.

Ten per cent of the sample locations in each group were selected randomly for measurement of physical parameters. The groups did not differ significantly in mean particle size, sorting coefficient or per cent mud, but did differ significantly in elevation. The lowest group was characterized by absence of the gastropod *Hydrobia totteni* Morrison, the next by presence of *H. totteni* and the polychaete *Scolelepis* (*Nerinides*) sp. I, and the highest by presence of *H. totteni* and absence of *S.* (*Nerinides*) sp. I. Differences in faunal composition in this area probably are caused by factors related to per cent tidal cover, such as temperature stress and time available for feeding.

Supported by a grant from the Whitehall Foundation to the MBL Systematics-Ecology Program; Systematics-Ecology Program Contribution No. 136.

Hypothermia, THAM and glucose in asphyxia of the newborn. James A. Miller, Jr., Faith S. Miller, Mickey Via and El Sayed H. H. Hegab.

Although exposure to cold elicits shivering and metabolism of brown fat in most conscious newborn animals, asphyxia blocks these reflexes. Consequently, cooling of asphyxiated animals prolongs survival. For most newborns 15° C. is the body temperature at which tolerance of asphyxia is the greatest. At this temperature puppies survive seven times as long as do their warm littermates.

Blood chemistry studies on newborn puppies showed that asphyxiation is accompanied by decreases in pyruvate and by increases in K, glucose, lactate, lactate/pyruvate ratio, and hydrogen ions. During asphyxia hypothermia (to 15° C. body temperature) reduces the rates of change in K to 1/3, in pH to 1/2, in glucose to 1/3, in lactate to 1/7, in pyruvate to 1/3 and in L/P ratio to 1/45 that in warm littermates. Since during asphyxia blood pH reaches levels which inhibit glycolysis, THAM (tris hydroxymethyl aminomethane) was used to maintain arterial pH and glucose was given either alone or with THAM to determine the effects of these compounds upon survival during asphyxia. In warm animals infusions (through the femoral vein) of THAM prolonged survival of puppies 17%; infusions of 5% glucose prolonged survival 19%; combining THAM and glucose prolonged survival 70%. Cooling to 15° C. body temperature prolonged survival to 655% and combining hypothermia with THAM-glucose infusions further prolonged survival to 1,104%.

Hypothermia is the most effective treatment tested thus far, but its efficacy is enhanced approximately 70% by THAM-glucose infusions.

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GENERAL SCIENTIFIC MEETINGS

AUGUST 21–24, 1967

Abstracts in this section are arranged alphabetically by authors. Author and subject references will be found also in the regular volume index, appearing in the December issue.

Annual reproductive turnover in the salt-marsh pulmonate snail, Melampus bidentatus. Martyn L. Apley, W. D. Russell-Hunter and Robert J. Avolizi.

Population dynamics, growth, and reproduction have been followed in a local natural population of *Melampus bidentatus* in 1964–67. The annual reproductive period always occurs between early June and mid-July, with normally three cycles of egg-laying. Each cycle has a definite semi-lunar periodicity with egg-laying strictly confined to four days in phase with the spring tides. Taking full or new moon as day 0, the patterned behavioral sequence involves aggregation (day −1), copulation (+1), egg-laying (+2 through +6) and dispersion (+6 through +8). Stocks of *Melampus* brought into the laboratory maintain the same semi-lunar pattern of reproductive behavior during the summer.

Reproductive turnover in terms of actual organic biomass was assessed. In 1967, wet weight, tissue dry weight, total organic carbon, and total nitrogen were determined for gonads. These were obtained immediately before breeding started (A), after the first cycle of egg-laying (B), and after the third cycle had concluded the annual reproductive period (C). Mean wet weights of individual gonads change from 6.32 mg. (A) to 6.95 mg. (B) and to 1.09 mg. (C). Dry weights are less variable, as are the organic carbon values, mean carbon being 698 μg. C (A), 845 μg. C (B) and 151 μg. C (C). Depletion of organic nitrogen is pronounced and uniform with mean values falling from 110 μg. (A) and 114 μg. (B) to 10 μg. (C). Actual egg production was most carefully assessed in 1966, when observed groups totalling 244 snails laid 219×10^4 eggs, then 345×10^4 eggs, and finally 254×10^4 eggs. Therefore, the overall fecundity totalled 818×10^4 eggs, corresponding to 33,150 eggs per standard snail per year. Mean biomass values for individual eggs are dry weight = 354 nano-g. and organic C = 109 nano-g. This average total egg output per individual corresponds to 7.3 mg. of dry organic material annually, while mean depletion of "standing biomass" in the gonads corresponds to 99.8 μg. N or 920 μg. of dry organic material from A to C. Such figures can be related to the mean growth increments for the standard snail. During the breeding season 87% of the non-respired assimilation (N-RA) is directed to reproduction. This corresponds to 46% of the total annual N-RA, or 32% N-RA if spring pre-breeding growth rates were sustained throughout the year.

Supported by Grant GM 11693 from the National Institutes of Health.

Microtubules and Golgi vesicles in cephalopod iridophore development. JOHN M. ARNOLD AND GEORGE SZABÓ.

The iridescence of *Loligo pealei* is caused by the presence of iridophores which are concentrated beneath the skin, around the ink sac, and particularly around the eye in the iris. The iridophores contain groups of reflective platelets making up multilayered iridosomes, each of which may be the product of one, two, or more cells. The iridosomal platelets are roughly 1000 Å in thickness, extremely electron-dense, and membrane-bound. Spaces continuous with the extracellular space occur between the individual platelets. In the adult iris most of the cytoplasmic region of the cell is occupied by the iridosome.

The embryonic development of the iridosomal platelets involves the fusion of vesicles derived from the Golgi apparatus to form the limiting membrane and the fusion of granules of ribosomal size to form at least part of the electron-dense platelet. Granules of the same density and staining characteristics are also found associated with the rough endoplasmic reticulum. Frequently a continuity of the Golgi apparatus, smooth and rough endoplasmic reticulum, and the nuclear envelope may be seen. In late embryos (Arnold stage 27 to 29) groups of microtubules can be seen to run unidirectionally into the ends of the developing platelets. Golgi-derived vesicles aline between these microtubules and fuse to form the limiting membrane. It is suggested that in this way the microtubules function in alinement of the Golgi vesicles and the Golgi apparatus gives rise to the plasma membrane.

This research was supported by National Science Foundation grant GB-5962 and National Institutes of Health grants HD-02668 and CA-05401.

Blood clot formation in the antenna of the hermit crab Pagurus longicarpus. FREDERIK B. BANG.

Direct observation of blood loss from the cut antenna of the hermit crab shows that the resultant clot has two and possibly three stages. The first consists of a sticking of morphologically unchanged amebocytes to the damaged antenna and to each other. This usually starts as soon as blood spills out into the surrounding sea water. The second stage—occurring two to ten minutes later—is a retraction of the mass into a smooth cap of cells, following which cell and fluid leakage stops. Finally the entire cell outline is lost, and the amebocytes within the antenna no longer stick. The first stage is inhibited or prevented by 0.01 *M* ethyl maleimide, or by injection of 0.002 *M* maleimide. The change in shape of amebocytes and the closure is inhibited by crowding under a coverslip and by 0.01 *M* cyanide. The lack of stickiness of the final clot was demonstrated by exposing a clotted antenna of one crab to the cells thrown out by the cut antenna of another crab. Times to closure of the cut antenna (bleeding times) support the above analysis.

The fixation and fine structure of white blood cells of the smooth dogfish shark, Mustelus canis. STEPHEN N. BARNES, ALLEN L. BELL AND SEYMOUR GELFANT.

Blood was withdrawn from the caudal vein and centrifuged at 3000 rpm for 15 minutes. Optimum fixation of the buffy coat was obtained with 5% glutaraldehyde for 90 minutes in 0.1 *M* Sorensen's buffer at pH 7.7 to which 216 grams per liter of sucrose had been added to make the final solution isotonic to elasmobranch blood plasma. The cells were then postfixed in 1% OsO_4 for 45 minutes and embedded in araldite.

The fine structural morphology of five types of white blood cells from *Mustelus* was described.

(1) The macrophages are characterized by the presence of parts of red cells in their cytoplasm, and by large vacuoles containing remnants of red cell membranes. The vacuoles are surrounded by the flat lamellar sacs of endoplasmic reticulum. The nuclei are multilobed. A unique feature of the macrophages are mitochondria in which the cristae are often seen to run parallel to the longitudinal axis.

(2) The granulocytes also contain a multilobed nucleus and are characterized by large, membrane-bound, non-crystalline, cytoplasmic granules. The endoplasmic reticulum is predominately of the agranular type.

(3) The lymphocytes are typified by their small amount of cytoplasm relative to the size of the nucleus. A large nucleolus is often seen. The mitochondria appear to be displaced to one end of the cell.

(4) The vacuolated cells have a relatively complex cytoplasm dominated by vacuoles of unknown function, which are frequently seen to join the plasma membrane of the cell, their lumen being open to the external environment.

(5) The plasma cells are characterized by a large amount of swollen ergastoplasmic sacs which contain a material of moderate electron density. Similar configurations are seen in vertebrate plasma cells.

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Effect of polyamines on ribosomal RNA synthesis in sea urchin embryos. C. BARROS AND G. GIUDICE.

Previous work has shown that in the sea urchin there is a sharp increase of r-RNA synthesis following gastrulation (Giudice and Mutolo, *BBA*, 1967, **138**: 276). Raina and Cohen (*P. N. A. S.* 1966, **55**: 1587) have shown that in *E. coli* there is a significant increase in RNA synthesis as a consequence of polyamine treatment. The present study was undertaken in order to study the effect of polyamines on sea urchin development.

Sea urchin embryos were cultured in Millipore-filtered sea water containing antibiotics. The experimental cultures contained also spermidine at a final concentration of 10^{-4}; 5×10^{-4}; 10^{-3} and 10^{-3} *M*. The rate of r-RNA synthesis was studied by continuous incorporation experiments with P^{32} from fertilization to blastula stage or from fertilization to early prism stage. Five-hour labelling experiments were done with H^3-uridine from two-cell stage to blastula; from hatching to early gastrula; and from early gastrula to prism stage. The RNA was prepared by the cold phenol method and analyzed by sucrose gradient. The specific activity of the 28S peak was chosen as a measure of r-RNA synthesis.

The results of the sucrose gradients show an increase of 26.14 (±6.8) in the spermidine-treated embryos over the controls in the specific activity of the 28S peak. The results also show that before hatching very little r-RNA synthesis takes place in both the controls and the spermidine-treated embryos. The 10^{-3} *M* concentration of spermidine was the most effective in the stimulatory effect of r-RNA synthesis. Putrescine seems to be less effective. Control experiments have shown that the increase of P^{32} incorporation into the r-RNA caused by spermidine can not be attributed to a simple increase in permeability. The total P^{32} uptake was in fact not increased in the presence of spermidine; nor was the incorporation into the total TCA-insoluble fraction.

Preliminary experiments in which the incorporation of H^3-uridine into the total RNA was measured after 10- and 20-minute pulses have suggested that this polyamine does not cause an increase in the overall RNA synthesis.

These data suggest that spermidine causes a specific increase in the rate of r-RNA synthesis in the stages from blastula to early prism.

This study was made in the Fertilization and Gamete Physiology Training Program (NIH grant T1 HD-26-06). Subsistence provided by the Population Council.

An analysis of the melanin-dispersing activity of aqueous and alcoholic extracts of eyestalks from the fiddler crab, Uca pugilator, by means of gel filtration and ultracentrifugation. CLELMER K. BARTELL, K. RANGA RAO AND MILTON FINGERMAN.

Fresh eyestalks were macerated in ethanol without excessive stirring. The alcohol-soluble fraction was centrifuged at low speed, evaporated at room temperature, suspended in distilled water, and passed through a 71 × 1.5 cm. column of Biogel P-6, eluting with distilled water. Aqueous extracts prepared similarly were also subjected to gel filtration. Two peaks of melanin-dispersing activity were obtained from both the aqueous and alcoholic extracts, at R_f-1.0 the activity varies and at R_f-0.6 the activity was generally high. The R_f-1.0 peak, always low in aqueous extracts, disappeared upon heating or vigorous stirring. The activity of the R_f-1.0 peak in the ethanol extract relative to the R_f-0.6 peak was higher and could be eliminated by initially stirring the alcoholic extract briefly. When the R_f-1.0 fraction was isolated and kept for 48 hours at 5° C., the biological activity was reduced and the resulting active material when re-chromatographed had an R_f of 0.6. The activity of the R_f-0.6 peak was not destroyed by these treatments. After alcohol extracts were resuspended in distilled water and centrifuged for two hours at 150,000 *g* the R_f-1.0 fraction was completely displaced to the pellet and the R_f-0.6 fraction remained in solution. When the extract was suspended in 1 *M* NaCl and centrifuged at high speed the R_f-1.0 fraction was present only in the supernatant material. The R_f-1.0 active material may consist of a lipoid moiety associated with an active peptide that is almost completely disassociated in water extracts. The appearance of the R_f-0.6 peak concurrently with the disappearance of the R_f-1.0 peak may be due to the release of a peptide from the lipoid component.

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The influence of fluphenazine and chlorpromazine on melanophore pigment-dispersing activity in the blood of the albino rat. JOHN M. BEDWINEK AND GEORGE T. SCOTT.

In the previous study, darkening was observed in white-background-adapted frogs on administration of ten phenothiazine ataraxics. The drugs had no effect on hypophysectomized frogs. An examination was made on the influence of two of these drugs, fluphenazine and chlorpromazine, on the level of pigment-dispersing activity in the blood of albino rats.

Extracts of blood samples were tested for pigment-dispersing activity by an *in vitro* frog skin bioassy in which hypophysectomized *Rana pipiens* were used. Hogben-Slome melanophore index readings were recorded both for blood extracts and for various concentrations of Pitressin to enable the activity of the extracts to be expressed in terms of equivalent Pitressin concentrations.

At the time of its maximum effectiveness, five hours after injection, fluphenazine at a dose of 20 mg./kg. increased the level of blood pigment-dispersing activity over controls by approximately one log unit Pitressin concentration. The minimum dose of fluphenazine producing this increase was 10 mg./kg., whereas the minimum dose of chlorpromazine producing the same increase was 50 mg./kg. The ratio of the minimum effective doses of these drugs as observed in the rat is similar to that observed in the production of pigment dispersion in white-background-adapted frogs.

Since certain phenothiazine derivatives have been reported to decrease the level of endogenous pigment-dispersing activity of rat pituitary tissue, it is proposed that the drugs cause an increase in the pituitary secretion of one or more of the three known pigment-dispersing polypeptides, MSH, MSH, and ACTH.

The research was supported by Grant MH 03903 from the National Institute of Mental Health and by an Undergraduate Research Participation grant from the National Science Foundation to Oberlin College.

Fine structure of the intestine and hemal sinuses of Thyone. ALLEN L. BELL AND A. FARMANFARMAIAN.

The hemal system of echinoderms consists of sinuses and lacunae of variable structure and unknown function. These channels are best developed in connection with the digestive tract of echinoids and holothuroids.

In *Thyone,* in addition to the mesenterial and antimesenterial channels which attend the length of the intestine, there is a network of channels interconnecting the looped regions of the first descending and the ascending parts of the intestine. The channels attending the length of the gut periodically send side branches into the gut wall, which has lacunar spaces in the connective tissue region containing a material similar in staining properties to that in the lumen of the channels.

Although there have been reports from light microscope studies of connections between the large main channels and the lacunar spaces of the gut, the precise relationship between the invading hemal branches to these spaces and the relationship of the lacunar spaces to the base of the gut endothelial cells have not been clarified. It is particularly interesting to clarify this point in the first descending part of the intestine since *in vivo* and *in vitro* experiments show that sugars are rapidly absorbed in this part of the intestine. Therefore, the fine structure of this region was examined using the electron microscope.

Serial sections 0.5 μ thick were cut through the entrance zone of a channel and examined in the light microscope. Adjacent thin sections were photographed using an electron microscope.

The results of this study demonstrate that the lumen of the hemal channel is continuous with the lacunar spaces in the connective tissue region of the intestinal wall. The basement membrane of the lacunae and the circular muscle of the intestine appear to pass into the wall of the hemal channel. Furthermore, there is an intimate contact of the material in the lacunar spaces of the intestine and the base of the endothelial cells of villus-like structures.

Experiments are in progress to assess the physiological significance of these findings.

Zooid response in Renilla. JOHN BUCK AND FRANK E. HANSON, JR.

When a dark-adapted colony of the alcyonarian *Renilla* is stimulated mechanically or electrically at any point on its upper surface (rachis) a wave of luminescence spreads radially at a constant velocity of 5–10 cm./sec. Localized electrical stimulation of siphonozooid clusters or individual autozooids elicits non-propagated flashes with latencies of about 10 msec., half-rise times of 15–20 msec. and half-decay times which may be as short as 60 msec. but are more usually hundreds of milliseconds.

With direct serial stimulation zooids respond to the first shock and usually fatigue rapidly: occasional mild summation can be ascribed to recruitment. Stimuli of certain strength × duration products applied to zooids as a single shock may excite zooids without causing a propagated rachidial wave of luminescence. When applied as multiple shocks (with constant voltage and proportionately reduced durations) zooid response fails and general rachidial waves begin. When zooids are excited *via* the normal pathway of the putative rachidial nerve net, they luminesce only after the second or subsequent shocks, facilitate markedly, do not fatigue easily, and can be excited by voltages that are sub-threshold when applied directly to zooids.

The frequency response limit of zooids is about 5/sec. Single autozooid tentacles, single autozooid calyx points and probably single siphonozooids can luminesce independently of adjacent parts.

The use of facilities of the Department of Invertebrate Zoology, MBL, and material and equipment of the Department of Biology, University of California, Santa Barbara, are gratefully acknowledged.

Chromosomes of Cyathura polita from Pocasset River, Massachusetts. MADELINE P. BURBANCK AND W. D. BURBANCK

Aceto-carmine squashes of young embryos taken from the marsupia of females collected in early June and fixed 5–12 hours in equal parts absolute alcohol and glacial acetic acid contained numerous nuclear division figures. Well-spread-out metaphase plates showed $2n = 44$. Pretreatment of young embryos with coumarin did not have any detectable effect on the spreading out of metaphase chromosomes.

We originally suggested an n number of 5 for *Cyathura polita* (then designated *Cyathura* sp.) and later tentatively revised the count to 12. These earlier counts were based on testicular smears fixed with Nissenbaum's and stained with Gomori's haematoxylin. Re-examination of these slides in the light of the current embryonic count reveals that the material is difficult to interpret because of clumping of chromosomes and the intensely dark haematoxylin stain. There are, however, clear counts of $2n = 44$ in the mitotic divisions of the spermatagonial cells and 22 units in the diakinesis stage of the first spermatocyte division.

It is planned to examine other populations of the isopod *C. polita* to determine if variation in chromosome number or morphology occurs throughout its range in estuaries from Maine to Louisiana.

This work was supported by National Science Foundation Grant GB-3122.

Activation of protein synthesis during maturation and fertilization in Asterias forbesi. GRACIELA C. CANDELAS AND ALBERTO MONROY.

Previous experiments in the organism have shown that there is no difference between the rate of protein synthesis between the fertilized and unfertilized eggs, at least up until the second polar body is excluded, and also it has been shown that permeability changes do occur as a result of fertilization.

Present experiments in which the ovaries were exposed to labelled amino acids and the eggs were obtained either by the use of the shedding stimulating factor or by spontaneous shedding indicate that the oocytes incorporate labelled amino acids into proteins to a variable extent in different animals. We have obtained values varying from 17 to 60% of the total uptake. The data also show that once a certain level of incorporation has been attained, no further change occurs as long as the germinal vesicle remains unbroken in the oocyte, and that the breakdown of the vesicle is followed by a marked increase in the incorporation which attains 95% of the total uptake. Fertilization which is carried out after the germinal vesicle breakdown does not elicit any change upon the rate of incorporation, at least up until the exclusion of the second polar body which is as far as these experiments have been carried out.

The incorporation of labelled amino acids into proteins occurs on polysomes. Some of the radioactivity in the heavy region of the gradients is not recovered in the monosome peak upon RNase treatment, but is recovered upon DOC treatment, thus suggesting that either the ribosomes in this peak are attached to membranes or the incorporation has occurred in membranes.

This study was made in the Fertilization and Gamete Physiology Training Program (NIH grant T1 HD-26-06).

Protein synthesis during maturation and after fertilization in the eggs of Cerebratulus lacteus. GRACIELA C. CANDELAS AND ALBERTO MONROY.

Eggs of *Cerebratulus lacteus* were loaded with H^3-amino acids by exposing pieces of the posterior end of females to sea water containing H^3-reconstituted protein hydrolysate for 2 hours. Germinal vesicle breakdown occurs within 15 minutes after shedding; the eggs can then be fertilized. The amount of labelled amino acids the eggs of various females take up is variable; between 20 and 40% of this is incorporated in hot-TCA-insoluble form. This percentage rises rapidly following germinal vesicle breakdown and then either slows down or stops altogether. No difference can be detected in the incorporation curves between unfertilized and fertilized eggs until the first polar body is given off by the fertilized eggs. On the other hand, the incorporation rises considerably in the fertilized eggs beginning at some time before the formation of the second polar body.

This study was made in the Fertilization and Gamete Physiology Training Program (NIH grant T1 HD-26-06).

Isotopic analysis of oogenesis in Habrobracon and Artemia. REV. J. D. CASSIDY, O. P. AND L. J. BEAUREGARD.

Experimental methods were developed to localize nucleic acids in the ovarian tissue of adult *Microbracon hebetor* and *Artemia salina.* Differentiating ova were incubated in tritium-labeled thymidine, uridine and actinomycin-D to study fluctuations during genetic transcription in both organisms. Dissected *Microbracon* ovarioles were cultured in isotopes prepared in Gibco Schneider's medium for 5, 10, 30, and 60 minutes. *Artemia* were treated in filtered sea water. The homogenates, tested by liquid scintillation, gave evidence of differential incorporation in the polynucleotides of the germarium, vitellarium and mature ova. Maximum uptake of the precursors, indicating regions of rapid synthesis, was traced to the germarium and vitellarium during the shortest treatment intervals. In the *Artemia* cells exposed to 2.5 μC./ml. of ^{3}H-actinomycin-D, maximum CPM were identified in the mature oocytes. This observation may account for the extreme sensitivity of brine shrimp eggs to this antagonist of the genetic code.

For correlated radioautography, slides with 0.5-μ sections of Maraglas were covered with either Kodak NTB-3 or Ilford 1–4 emulsions, developed after 20 days of exposure at 5° C. and stained with azure B. The highest thymidine grain counts were found in the follicle and nurse cell nuclei, the accessory nuclei of the oocyte and the oocyte-follicle cell interface. These data lend support to the derivation of accessory nuclei from the follicular epithelium. Uridine was concentrated over the nuclei of the nurse cells. Appropriate enzymatic extractions were conducted on adjacent sections. The precise role of the extra-germinal vesicle DNA in the encoding of developmental information remains to be investigated.

Supplementary findings were derived from these experiments. The cytological fine structure was normal throughout the *in vitro* incubation of the dissected *Microbracon* ovarioles. With high resolution electron microscopy synaptinemal complexes were resolved in early yolky oocytes of *Artemia.*

RNA synthesis in non-nucleate fragments of Arbacia eggs. JOHN CHAMBERLAIN.

Recent work has demonstrated the presence of DNA in the cytoplasm of eggs of many species. It was thus of interest to investigate the RNA-synthesizing capacity of non-nucleate fragments of eggs of the sea urchin *Arbacia punctulata.* Eggs were shed into Millipore-filtered artificial sea water buffered with 0.01 *M* Tris, pH 8.2. The eggs were washed in this medium containing penicillin (100 units/ml.), streptomycin (250 μg./ml.), and sulfadiazine (50 μg./ml.). They were then split into nucleate and non-nucleate fragments by centrifugation in four-step sucrose gradients. All sucrose solutions were buffered with 0.01 *M* Tris, pH 8.2, and contained antibiotics. The resulting bands of nucleate and non-nucleate fragments were collected, washed in artificial sea water, activated with hypertonic sea water, washed and incubated in the presence of 5-H^3-uridine (24.7 mc./μM, 20 μc./ml.). The fragments were then washed in cold acid sea water, pH 4.8, and lysed in 0.01 *M* acetate buffer containing 0.5% SDS, pH 5.1. The lysates were extracted once with cold phenol, dialyzed against acetate buffer, treated with the enzymes DNase, pronase, and, in certain experiments, RNase. They were then precipitated with ethanol, the precipitates taken up in Tris-SDS buffer, pH 7.5, and layered on Tris-SDS sucrose gradients (15–30% w/v). The gradients were spun for 12 hours at 24,000 RPM. Fractions were collected and assayed for absorbance at 260 mμ and TCA-insoluble radioactivity.

Such analysis indicates that activated non-nucleate egg fragments incorporate uridine into heterogeneously sedimenting material which is heavier than transfer RNA. This material is sensitive to RNase, insensitive to DNase and pronase, and is insoluble in cold 5% TCA. Comparison of the sedimentation profiles of material extracted from activated non-nucleate fragments with those from activated nucleate fragments and activated and fertilized whole eggs indicates that the synthesis in non-nucleate fragments accounts for a large portion of the synthesis in whole eggs and nucleate fragments.

This study was made in the Fertilization and Gamete Physiology Training Program (NIH grant T1 HD-26-06).

Observations on the reproductive and general morphology of Streblospio benedicti Webster. MAJORIE COLLIER AND MEREDITH L. JONES.

Females of this spionid polychaete, which brood their young in dorsal pouches, were fixed in Bouin's fluid, Zenker's fluid, or glutaraldehyde, serially sectioned at 5 micra, and stained with Mallory's triple stain or hematoxylin and eosin.

The bilateral, interramal pouches were usually found on the twenty-second through the thirty-fifth setigers. The genital strands, with oocytes up to 225 micra in diameter, extended from the twelfth through the last pouch-bearing setiger.

In looking for the way in which the eggs move from the coelom to the dorsal pouches we found that there were fairly complete, if not complete, septa and that the pouches were not merely epidermal flaps over the dorsum but were thin-walled, dorsolateral extensions of the coelom. The youngest occupant of a pouch was an uncleaved egg, the oldest, a 12-setiger larva. How the eggs move from the anterior segments in order to get to the region of the pouches was not discovered.

From 2 to 5 seminal receptacles were found in the females, one per setiger, beginning about the fourteenth setiger. In other spionids seminal receptacles are dorsally located; in *Streblospio* they are between the neuropiles of the ventral nerve cord. There is a duct for each receptacle, ventral to the nerve cord, leading from the outside to the anterior of the receptacle. All but one receptacle contained masses of sperms.

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Oxygen equilibria of hemocyanin. HENRY A. DEPHILLIPS, JR. AND K. E. VAN HOLDE.

The oxygen binding properties of the hemocyanin obtained from the squid, *Loligo pealei,* have been studied. Dilutions of the serum into 0.1 ionic strength (0.125 for those containing Mg^{++} ion) buffers were made in every case. Solutions contained about 2.5 to 3.0 mg./ml. of protein. Oxygenation was achieved by the use of a tonometer with an attached 1-cm. quartz cuvette. The increase in the optical density at 345 $m\mu$ was used to follow oxygen uptake. After the addition of an oxygen increment, solutions were allowed to equilibrate in a thermostatted shaker bath at 25° C. In the pH range 6.24 to 8.44 in the presence of 0.01 M Mg^{++} ion under all conditions examined, the 59S molecule of molecular weight 3.8 million is the predominant species. Oxygenation over the pressure range of less than one millimeter to approximately one atmosphere of pure oxygen shows a gradual transition from non-cooperativity at low pH to increasing site-site interaction in the pH range 7.0 to 8.0. Above pH 8.0, the slope of the Hill plot is greater than 1.0 over the entire range of oxygen pressures used. The 19S subunit of molecular weight 770,000 is obtained above pH 7.2 in the absence of Mg^{++} ion. Oxygenation of this subunit at pH 7.75 shows behavior similar to the 59S molecule at this pH. Finally, above pH 9.5 an 11S subunit of molecular weight 375,000 is obtained. Oxygenation at pH 10.45 yields a Hill graph whose slope decreases to a value less than one. Such behavior would be expected for negative site-site interaction or, possibly, for a molecule containing two types of binding sites, one, strong oxygen binders, the other, weak. This system exhibits a normal, though very large, Bohr effect. Fifty per cent oxygenation is obtained at about 1000 mm. Hg at pH 6.24 whereas only about 10 mm. Hg are required for 50% saturation at pH 8.44.

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Increased potassium flux in the lobster walking limb axon induced by hyposmotic challenges. PHILIP B. DUNHAM, ALAN R. FREEMAN AND HARRY GRUNDFEST.

Motor axons of lobster swell about 70% when the standard bathing medium is made hyposmotic by reducing NaCl to 30%. In a Cl-free medium with an impermeant anion (propionate) substituted, the swelling in response to the hyposmotic challenge is $<10\%$. When returned to the respective isosmotic media, the fibers in Cl-saline shrink about 15% below the control value but those in propionate saline shrink about 55%. When the bathing medium is sucrose or Tris-Cl hyposmotic challenges also induce the smaller swelling and the larger subsequent shrinkage but isosmotic substitutions of NaCl with Na-propionate, Tris-Cl, or sucrose are without significant effect. Thus, a hyposmotic challenge appears to be the effective stimulus for the anomalous volume changes in this Cl-free media. The maximum amount of shrinkage was dependent upon the magnitude of the challenge. The level of steady-state shrinkage varied with the duration of exposure to the hyposmotic medium and indicated the time course of solute efflux. The changes in membrane potential (depolarization) further indicated that loss of a K salt could account for most of the efflux. Tracer measurements on small nerve bundles (two to six fibers) support this conclusion. The hyposmotic challenges increased K^{42} efflux to an initial peak 10-fold the resting value and the efflux remained high. A net loss of K^+ was demonstrated by equilibration experiments with K^{42}. The smaller shrinkages observed in the NaCl salines denote a two-way movement of K^+ and therefore indicate an increase in K^+-permeability, while the larger loss in Na^+-free and/or Cl^--free hyposmotic media signifies that these conditions induced a net K^+ efflux. The tracer measurements correlated well with the estimates of solute efflux based on volumetric and electrophysiological data. Up to about 60% of the contents may be lost by the axon. While there is sufficient K^+ in the axon to account for the loss of cations, intracellular Cl^- is probably far too low to furnish all but a small part of the anion.

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Collagenolytic activity in crustacean hepatopancreas. Arthur Z. Eisen.

An active collagenolytic enzyme capable of degrading native collagen at physiological pH and temperature has been demonstrated in the crustacean hepatopancreas. The enzyme is present in the "spider crab," *Libinia emarginata,* the "fiddler crab," *Uca pugilator,* and the common "green crab," *Carcinus maenas.* The "square backed fiddler crab," *Sesarma reticulatum,* has only minimal enzyme activity, perhaps related to the reluctance of these crabs to feed under laboratory conditions.

Explants of hepatopancreas cultured on native collagen gels liberate a collagenolytic enzyme capable of producing almost total lysis of the gels within 24 hours. Bacterial cultures for both aerobic and anaerobic organisms showed no growth on numerous different occasions. Hepatopancreas frozen and thawed a minimum of 6 times was still capable of producing lysis of collagen gels. This is in marked contrast to the collagenases from other animal sources which require living explants for substrate lysis. This suggests that the collagenolytic enzyme may be stored in the active state in the cells of the hepatopancreas and perhaps released when needed, while other animal collagenases appear to be synthesized *de novo.*

Of importance to the characterization of this enzyme is the fact that an active preparation can be extracted from fresh tissue. Thus the collagenolytic enzyme from crab hepatopancreas is unique, since tissue extracts from other animals have consistently failed to yield an enzyme which will attack native collagen at neutral pH and physiological temperature. A 0.2% solution of crude enzyme reduces the specific viscosity of collagen solutions by approximately 80% in 3 hours at pH 7.4 and 25° C. An initial rapid decrease in specific viscosity occurs during the first 10 minutes of the reaction with a subsequent slower decline. The crude enzyme is not inhibited by EDTA, thus differing in this respect from other known collagenases. It is inhibited by p-tosyllysylchloromethane and by soybean trypsin inhibitor. A 6- to 8-fold purification of the enzyme is obtained following column chromatography on Sephadex G-150.

Fellow of the Medical Foundation, Boston, Mass.

A possible mechanism for the actions of the red pigment-concentrating and red pigment-dispersing hormones in the prawn, Palaemonetes vulgaris. Milton Fingerman, P. M. Connell and Paul Yoshioka.

The red pigment-concentrating hormone (RPCH) has a sodium requirement for maximal activity whereas the red pigment-dispersing hormone (RPDH) has a calcium requirement. The response to a constant amount of each hormone is proportional to the concentration of the corresponding cation up to the isotonic concentration, the highest used. Two drugs known to alter sodium movement across membranes were applied *in vitro* to red chromatophores with maximally dispersed pigment to determine whether they affect the response to RPCH. Ouabain in a concentration of one milligram per milliliter of isotonic sodium chloride applied at the same time as the hormone inhibited the response by 42%, but when applied 30 minutes prior to the hormone inhibited by 75%. In contrast, tetrodotoxin enhanced the response to RPCH. A solution containing 10^{-5} of a gram of tetrodotoxin per milliliter enhanced the response 52%. These observations are consistent with the following hypothesis. Sodium ion is actively pumped out of the chromatophore by a ouabain-sensitive mechanism, but enters through pores that can be plugged by tetrodotoxin. RPCH either stimulates the sodium pump or blocks the influx of sodium, resulting in a greater differential between the sodium concentrations inside and outside the chromatophore. This heightened difference is the signal which in some manner activates the pigment-concentrating mechanism. On the other hand, RPDH could inhibit sodium transport while increasing the permeability of the cell membrane to calcium. Increased internal calcium would not only activate the pigment-dispersing mechanism but also would further inhibit sodium transport, thereby shutting off the pigment-concentrating mechanism while allowing the pigment-dispersing mechanism to function.

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New observations on the feeding mechanism in Lineus ruber (Rhyncocoela). FRANK M. FISHER, JR. AND NANCY M. CRAMER.

Since members of the phylum Rhyncocoela have a complete digestive tract it is often reported that they obtain their nutrients by actively eating small invertebrates. It has also been reported that the eversible proboscis of the nemertines is used to entrap prey subsequent to engulfment *via* the mouth. Our observations on *Lineus ruber* do not totally support these notions.

While small specimens (15.0 ± 1.9 mgm. wet weight) of *L. ruber* do orient toward dead or decaying portions of various invertebrates, little or no interest is exhibited toward living specimens. Further, we have not observed the action of the proboscis in any feeding mechanism.

Orientation to dead or decaying animal material is mediated *via* small dialytic materials. Of 20 amino acids tested, we have demonstrated that the amino acids, taurine and histidine, and the imino acid proline are capable of eliciting a direction-oriented response. In addition, glucose and its aceylated amino derivative, n-acetyl-glucosamine, as well as cellobiose provide a similar reaction.

We have also demonstrated that *L. ruber* can accumulate L-alanine, L-isoleucine, and L-aminocyclopentane-1-carboxylic acid (cycloleucine) and glucose against a concentration difference when these substrates are provided at concentrations resembling those found in sea water, *i.e.*, $10^{-6}\,M$ or less. The permeation process follows an adsorption isotherm and competitive inhibition has been demonstrated within each of the classes of compounds. The concentration of freely extractable compounds inside the nemertine body ranges from 10 to 1200 times that in the surrounding medium. Electron microscopic examination of *L. ruber* reveals that there are abundant microvilli interspersed between cilia on the epidermal surface. Such cell amplification is consistent in theory with the absorptive process. This is the first report of: (1) the active absorption of organic molecules by a nemertine, and similarly (2) the identification of organic molecules which serve as the stimulus for food location.

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Studies on the nematocyst capsule protein from the sea anemone Metridium marginatum. LOUIS FISHMAN AND MILTON LEVY.

The capsule proteins of nematocysts from various metazoa have been reported to contain hydroxyproline. A study was undertaken to establish the nature of this protein. Undischarged nematocysts, obtained by homogenizing whole *Metridium marginatum,* followed by differential centrifugation in $1\,M$ sucrose sea water, were discharged in distilled water. The capsules were resistant to digestion by trypsin, Pronase, elastase, and bacterial collagenase, and did not dissolve in 8 *M* urea, 5 *M* LiCl, 0.5 *M* acetic acid, nor in 1% sodium dodecyl sulfate (SDS). They could be dissolved only with disulfide reducing reagents such as mercaptoethanol and dithiothreitol (DTT). A more homogeneous preparation was obtained by collecting the acontia from anemones, discharging the nematocysts in 0.05 *M* Na EDTA containing 0.2% SDS, washing, digesting with trypsin (1 mg./ml.), washing, extracting with 0.0065 *M* DTT in pH 7.75 0.1 *M* Tris buffer for 1 hour, exhaustively dialyzing the extract, and finally lyophilization.

This material gave a single peak with a trailing edge in the ultracentrifuge using a synthetic boundary cell and an S_{20} of 3.8 which corresponds to a molecular weight of about 50,000. Amino acid analysis of a hydrolysate gave the following composition given as residues per 1000 total residues; hypro 69.8; pro 143.6; gly 202.8; asp 47.8; thr 22.8; ser 56.5; glu 108.7; ala 80.4; val 25:7; met 36.5; iso 8.2; leu 25.5; tyr 20.8; phe 17.6; lys 38.0; his 16.0; arg 23.8; OH lys 15.7; cystine/257.3. This protein resembles invertebrate collagens. Its resistance to solution is probably due to extensive intermolecular disulfide crosslinking.

Tests for the effects of antibodies on the acrosome reaction in Arbacia punctulata. C. R. FOURTNER AND C. B. METZ.

The acrosome reaction can be initiated in sea urchin sperm by high pH (Dan, 1952) or egg water treatment (Collier, 1959). To examine for effects of pretreatment with antibody, 0.5% sperm suspensions were mixed with control, antisperm and anti-egg jelly rabbit gamma globulin. After five minutes the suspensions were treated with Tris-buffered sea water (final pH of mixture 9.0 ± 0.4) or egg water to initiate acrosomal reactions. Two minutes later formalin (8% final concentration) was added to fix sperm. Whole mounts were prepared and examined with electron optics. Fifty sperm per grid on two or more grids were examined for each preparation.

In these different experiments 80 to 95% of sea water, control or antibody gamma globulin pretreated sperm had reacted acrosomes following high pH treatment. Less than 10% of sperm in samples maintained at pH 8 had reacted acrosomes.

Egg water was used to initiate the acrosome reaction in additional experiments. Sixty-five to 80% of control gamma globulin pretreated sperm reacted. Thirty-five to 50% of antisperm (6 experiments) or anti-egg jelly (4 experiments) antibody pretreated sperm had acrosome reactions; 55 to 80% of sea water pretreated sperm reacted. Less than 10% of control sperm treated with control or antibody gamma globulin but not with egg water reacted. The apparent decrease in acrosome reactions in the antibody pretreated samples is believed to result from irreversible agglutination. Many agglutinated sperm were distorted or broken at the anterior end. The latter were classed as unreacted sperm but were probably reacted but damaged sperm.

It is concluded that the antibody treatment neither facilitates nor inhibits alkali or egg water initiated acrosome reactions in *Arbacia punctulata.*

This study was made in the Fertilization and Gamete Physiology Training Program (NIH grant T1 HD-26-06).

Alterations in the malate dehydrogenase isozyme pattern during early development in Arbacia embryos. RALPH P. FRANCESCONI, M. MICHAEL THALER AND CLAUDE A. VILLEE.

By means of polyacrylamide disc gel electrophoresis, L-MDH isozyme patterns were established for both soluble and particulate malate dehydrogenase in both egg and sperm samples collected from *Arbacia punctulata.* The unfertilized eggs displayed eight bands of MDH activity spread throughout the gel column while the sperm sample produced three isozymes of relatively equal anodic migration. In zygotes collected just five minutes after fertilization, three anodic bands appeared corresponding precisely to the three isozymes common to spermatozoa. In this area, egg bands were evidently masked by the rapidly developing spermatozoan isozymes due to the extremely high specific activity of spermatozoan MDH. Upon increased incubation times lighter cathodal bands corresponding to the unfertilized egg appeared.

After a 30-minute development *Arbacia* embryos displayed three anodal bands and two more lightly staining cathodal bands not noticeably different from the zymogram pattern displayed at five minutes after fertilization. However, fifty minutes after fertilization the pattern was altered strikingly with three darkly staining bands apparent between the anodal and cathodal bands which appeared in the five-minute and 30-minute embryos. Ninety minutes after fertilization, these three bands were of equal intensity with the darkest bands on the gel column, and the pattern was such that between 30 and 90 minutes there occurred a distinct increase and intensification of bands in the anodal portion of the gel column. At later time intervals studied (*i.e.,* two hours, 14 hours, 24 hours) the isozyme pattern seemed to revert back to a stabilized pattern with distinct anodal and cathodal components, as opposed to the type of continuum observed at 50 and 90 minutes after fertilization.

Specific activity measurements of total MDH indicated marked changes during the first forty hours of development.

Microspectrophotometry of single crustacean rhabdoms. Timothy H. Goldsmith, Andrew E. Dizon and Hector R. Fernandez.

Single rhabdoms of dark-adapted prawns (*Palaemonetes vulgaris*) were isolated under near infrared light and examined with lateral illumination, using a dual beam recording microspectrophotometer. Individual rhabdoms contain two photosensitive pigments with λ_{max} at 555 and 496 nm. Using a 4-μm. test spot, we have recorded absorption spectra in the region 370–700 nm. without producing significant bleaching of either pigment. At the λ_{max}, P_{555} absorbs 3–4% of the incident light, and P_{496} about half that. On exposure to bright red light (90 sec., Corning filter 2-59, λ's longer than 620 nm.) at pH 6.6, P_{555} is converted into a long-lived (stable for many minutes or even hours at 24° C.) metarhodopsin with λ_{max} at 496 nm., but distinct from P_{496}. The molar extinction of this metarhodopsin is greater than the parent pigment. Subsequent exposure to bright yellow light (90 sec., Corning filter 3-71, λ's longer than 470 nm.) bleaches P_{496}—but not the metarhodopsin previously formed—and gives rise to no detectable photoproduct in the spectral region examined. As revealed by difference spectra, P_{496} is described fairly well by Dartnall's empirical nomogram of vertebrate visual pigments, but the absorption spectrum of P_{555} is significantly narrower. Comparison of these difference spectra with the spectral sensitivity of *P. paludosus* determined electrophysiologically suggests that both P_{555} and P_{496} are visual pigments.

Crustacean rhabdoms consist of microvilli arrayed at right angles to the axis of the rhabdom. The microvilli are organized in layers which are stacked along the axis of the rhabdom. Each layer is several μm. thick, and the microvilli of alternate layers are oriented at right angles to each other. Experiments with narrow slits (1×9 μm.) oriented transversely with respect to the long axis of the rhabdom indicate that both pigments occur mixed within a single layer of microvilli.

This work was supported in part by USPHS grant NB-03333.

Melanogenesis in the fiddler crab. Jonathan P. Green.

Autoradiographic studies of the incorporation of ^{3}H-tyrosine into the melanin of *Uca pugnax* indicate that pigment synthesis occurs only in the melanophore. Silver grains can be seen overlying even the finest processes of melanophores with dispersed pigment, while grains are clustered over the cell center in melanophores with concentrated pigment. The results confirm my earlier finding that melanin synthesis is independent of the state of pigment dispersal.

An *in vitro* radiometric analysis for crab tyrosinase activity has been developed, and basic parameters of enzyme activity (*e.g.,* optimum pH [7.8] and optimum temperature [22° C.]) have been determined. This technique shows that epidermal extracts catalyze the incorporation of 30 times as much ^{14}C-tyrosine into melanin as do comparable extracts of muscle. Epidermal extracts of *Uca pugnax* incoporate four times as much ^{14}C-tyrosine into melanin as do extracts from *Uca pugilator*. Variations in tyrosinase activity correlate both with the molt cycle and with diurnal variations. The enzyme activity falls into distinct classes which may reflect specific stages in epidermal metabolism prior to ecdysis.

The idea that melanogenesis is in some measure correlated with the molt cycle comes from a variety of observations. An animal in the "pre-molt" stage will have molting accelerated by eyestalk removal whereas animals in other stages may not. I previously noted that destalked crabs incorporated up to 70% more ^{14}C-tyrosine into melanin than did entire animals. Present work suggests that eyestalk ablation may not lead to precocious molting, and that the effect of eyestalk removal varies with the animal's position in the molt cycle. Only in those animals in a "pre-molt" condition does eyestalk ablation lead to a significant increase in ^{14}C-tyrosine incorporation into melanin. Significant differences in tyrosinase activity from entire and destalked crabs have been obtained.

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Further observations on the functional organization of the crustacean proprioceptor, the propus-dactylus organ. H. BERNARD HARTMAN AND EDWARD G. BOETTIGER.

We have recently reported that in the propus-dactylus organ of the crab *Cancer irroratus* Say, receptor cells are segregated according to function. The dendrites of relaxation-sensitive cells (RSC) are inserted into the dorsal side of the elastic strand, while those of elongation-sensitive cells (ESC) insert into a narrow band on the anterior side. Small neurons inserted distally along the anterior side near the ventral edge produce tonic responses to static positions. In this communication we report our analysis of the response characteristics of the movement neurons (RSC and ESC).

The elastic strand was lengthened and shortened by means of a piezoelectric transducer which produced linear excursions of up to 200 μ, and velocities from 25 to 400 μ/sec. Impulses from axons were monitored using a suction electrode during imposed movements at the 0° (open), 45° (rest), and 90° and 100° (closed) regions of the movement arc.

The distal RSC signal dynamic position and velocity in open arc region, while medial to proximal RSC are only capable of signaling opening direction. Medial ESC, in addition to providing direction information, also indicate velocity as the dactyl is being closed. However, the proximal ESC signal direction, velocity, and dynamic position information in the closed arc region.

These results provide a substantial clue as to how the crab maintains the dactyl at the rest position of 45°, and pinpoints specifically which neurons are responsible for providing this negative feedback information. What structural relationships exist between the elastic strand and the dendrites which break up information into such distinct and separate channels?

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Effect of temperature acclimation on the protein synthetic system of the toadfish. AUDREY E. V. HASCHEMEYER.

Toadfish acclimated to a temperature of 10° for one week show significantly increased liver protein synthesis at 20°, about two-fold, compared to control fish maintained throughout at 20°. Incorporation into protein was determined after 15–30-minute incubation periods with C^{14} amino acids injected into the dorsal artery.

In vitro protein synthesis by toadfish liver homogenates without added synthetic messenger RNA showed a normal temperature dependence over 10°–30° ($Q_{10} \sim 2.5$). This system, however, is too crude for comparison of activity levels among different groups of fish. Sucrose gradient analysis of liver post-nuclear supernatant indicated that toadfish and other fish livers (sea robin, tautog) do not have a significant pool of free ribosomes; thus the observed increase in protein synthesis *in vivo* cannot be attributed to conversion of inactive ribosomes to active polyribosomes utilizing new or pre-existing messenger RNA.

Preparations of aminoacyl-sRNA synthetases (amino acid activating enzymes) from livers of control (20°) fish and fish acclimated at 10° for two weeks were assayed with phenylalanine alone or a mixture of 15 amino acids, using a highly purified rat liver sRNA preparation. Kinetic data were collected as a function of enzyme concentration and temperature of incubation. Similar studies were carried out on the stimulatory activity of the 100,000 *g* supernatant containing the transfer enzymes required for protein synthesis in a system consisting of toadfish ribosomes, polyuridylic acid, and aminoacyl-sRNAs. Activating enzymes from control and cold-acclimated fish were identical, both in activity per mg. of protein and in temperature coefficients *in vitro* ($Q_{10} \sim 1.1$–1.5 in the 15°–35° range). Transfer enzyme activity, however, was about 75% greater in cold-acclimated fish. It will be important to determine if this is the rate-limiting step *in vivo*. Further purification of the enzymes for *in vitro* assay also will be carried out.

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Effect of papain-digested anti-jelly antibody on the jelly-coat and fertilizability of Arbacia eggs. NATILLE HEADRICK AND CHARLES B. METZ.

Treatment of *Arbacia* eggs with rabbit anti-*Arbacia* egg jelly antibody precipitates the egg jelly and inhibits fertilization. Sperm penetration could be prevented by the mechanical barrier created by the precipitated jelly. This and other possible secondary effects due to the cross-linking of antigens by the bivalent antibody might be eliminated by use of a univalent form of the antibody.

Such univalent antibody was prepared by papain digestion (Porter, 1959) and its presence demonstrated as follows: (1) Ouchterlony plates pretreated with papain-digested anti-egg jelly antibody failed to produce precipitin bands when undigested antibody was diffused against egg jelly solution (2) papain-digested anti-egg jelly antibody-pretreated *Arbacia* eggs wrinkled following exposure to goat anti-rabbit globulin (Coomb's test).

Eggs treated with anti-egg jelly antibody for 30 minutes lack a jelly layer. The removal of the jelly by the univalent antibody treatment contrasts with the jelly-precipitating action of the bivalent antibody. In fertilization tests eggs treated with univalent anti-egg jelly antibody were not significantly different in fertilizability from eggs treated with papain-digested control globulin (eight experiments, two bleedings from rabbit #6612) on eggs previously dejellied by acid sea water (five experiments). Eggs treated with the bivalent anti-egg jelly were not fertilizable. Eggs which were first treated with univalent anti-egg jelly antibody and subsequently exposed to goat anti-rabbit globulin (Coomb's test) were reduced in fertilizability—however, not to the extent achieved by bivalent antibody (two experiments).

To further investigate the reaction between anti-egg jelly antibody and jelly the antibody was tested for its effect on the sperm-agglutinating action of jelly solutions. The bivalent antibody reduced the agglutinating action of the jelly solution, but the univalent antibody failed to do so. Evidently, the anti-egg jelly antibody does not block the sites of the jelly molecule that react with sperm to produce agglutination.

This study was made in the Fertilization and Gamete Physiology Training Program (NIH grant T1 HD-26-06).

Ultrastructural studies of sperm and sperm-egg interaction following natural fertilization of Libinia emarginata. GERTRUDE W. HINSCH.

The non-flagellate sperm of *Libinia emarginata* has a large spherical acrosome with three concentric layers of varying electron density. Except for the apical cap region, the acrosome is surrounded by a membranous lamellar system and is embedded in the nucleus. Three arms and a posterior process of nuclear origin project out from the body of the sperm. These processes contain a microtubular system. Two centrioles are present in the mature sperm. Spermatophores containing sperm are transferred to the seminal receptacles of the females at mating.

To study early stages of sperm-egg interaction following natural insemination, eggs were collected and fixed during and following spawning. Fertilization is internal and naturally-spawned eggs have numerous sperm penetrating the chorion. The chorion has at least two layers, a thin dense outer layer and a thick spongy inner layer. At fertilization, the apical caps of the sperm attach to the egg chorion, the acrosomal region penetrates the chorion and the sperm enter the perivitelline space. Penetration of the chorion may involve lysins.

Fertilization and egg attachment to the brood pouch occurs during the first two hours after oviposition. An egg membrane complex is formed by the extrusion of materials from the egg cell surface.

This study was made in the Fertilization and Gamete Physiology Training Program (NIH grant T1 HD-26-06).

Electrical activity of isolated parts of Tubularia. FLORENCE HOFMAN AND NORMAN B. RUSHFORTH.

Tubularia possess several sets of pacemaker systems. Electrical potentials produced by these systems may be recorded externally from different parts of the intact animal. This study was undertaken to determine which structures, when isolated from the polyp, spontaneously produce potentials and which are electrically quiet.

Excising the hydranth from the neck region separates the hydranth and neck pacemaker systems. Potentials (Neck pulses, up to 5 mV.), in various temporal patterns, were observed in reçordings of several hours from the neck region of isolated stalks. Bursts of Neck pulses are associated with neck contractions.

Hydranths isolated from the stalk gave large potentials (Hydranth pulses, up to 5 mV.) as single events or in bursts. Such pulses were correlated with "concerts" which are characteristic of the intact polyp. Smaller potentials (Proximal Tentacle pulses, up to 100 uV.) occurring predominantly in bursts were also observed in hydranth records. Proximal Tentacle pulse bursts are suppressed in the initial portion of the hydranth inter-burst cycle, and gradually increase in the later phases of the cycle. Isolated parts of a proximal tentacle spontaneously produce potentials, showing that portions of such tissue are capable of packmaker activity. Isolated distal tentacles give pulses which are associated with movement of the tentacle tip.

Immature gonophores replicate pulses produced by the attached hydranth, but when isolated give no observable potentials. In contrast, mature gonophores produce potentials in addition to conducted Hydranth pulses. Isolated mature gonophores give potentials in various temporal patterns, implying endogenous pacemaker activity.

These results confirm that *Tubularia* has several distinct pacemaker systems. Such systems exhibit various interactions in the intact polyp which underly its coordinated behavior.

This research was supported in part by the National Science Foundation and the National Institutes of Health.

Effects of detergents, fluorescent dyes and sulfhydryl reagents on some of the membrane properties of the squid axon. F. C. HUNEEUS AND H. L. FERNANDEZ.

Loligo pealei axons were perfused by the double cannulation procedure. Reagents applied internally were dissolved in a standard medium (200 m*M* KF, 200 m*M* K-glutamate and sucrose, pH 7.3.). External medium was natural sea water. Axoplasm was removed by perfusing Pronase 0.01% for 1 minute.

The anionic detergents: N-methyl-n-oleyl taurate, dodecyl sulfate, dodecyl sarcinate and decyl betaine, 0.3 m*M*, block the action potential and increase the membrane relative conductance within 5 minutes. Both effects could be reversed in the last two mentioned if rapidly washed with standard medium. They are approximately ten times more active on the inside. The cationic detergent hexadecylpyridinium, 0.3 m*M*, blocks with a marked increase in conductance, irreversibly and on either side of the membrane. The non-ionic polyoxyethylene-polyoxypropylene failed to have an effect.

The fluorescent dyes: rhodamine 6-G and auramine 0, 0.2 m*M*, block reversibly within 2 minutes without affecting the membrane conductance. Externally their effect is slower and irreversible. Rhodamine B has the same effects but is less active and can be detected in the perfusion fluid shortly after external application.

The sulfhydryl reagents, P-hydroxymercuribenzoate and mercuric chloride 1 m*M* produced an increased membrane relative conductance simultaneous to the action potential block. Both effects could be reversed with mercaptoethanol, 30 m*M*. N-ethyl maleimide, 2m*M*, and dithiobisnitrobenzoate (Ellman's reagent), 1 m*M*, had similar effects, although irreversible. The transient hyperpolarization described for the recovery of a mercury-blocked axon upon addition of mercaptoethanol was found to be an artifact produced by the local interaction of mercury, thiol and the platinum tip of the electrode. Tetranitromethane, 0.8 m*M*, also blocked the action potential with an increased membrane conductance, irreversibly when applied to either side of the membrane. It is probably interacting with sulfhydryl groups in the membrane.

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Analysis of burrowing mechanism in Leptosynapta tenuis and Golfingia gouldi. R. DOUGLAS HUNTER AND HUGH Y. ELDER.

In the Woods Hole region the holothurian *Leptosynapta* and the sipunculid *Golfingia* are found burrowing in the same intertidal muddy-sand flats. However, their burrowing mechanisms differ markedly.

Behavior was studied by observation and photography of animals burrowing in a chamber of sand between glass plates. To record coelomic pressures while above or below the sand surface, animals were cannulated to a Statham diaphragm transducer and polygraph.

In Golfingia the burrowing cycle consists of everting the proboscis into the substrate, mushrooming the tip and drawing up the rest of the body to this "anchor." *Leptosynapta* burrows by scraping sand laterally with the oral tentacles while the body advances by means of anti-peristaltic locomotor waves. This phase alternates with thrusts by the animal against the sides and head of the burrow, probably to compact the walls with mucus and loosen the sand ahead.

Coelomic pressure records in *Golfingia* show that the animals develop a high pressure (equivalent to 150–250 cm. of distilled water) only during proboscis protrusion (and 2–20 cm. during other phases). Occasionally *Golfingia* burrowed successfully utilizing much lower pressures (maximum less than 50 cm.) and it is suggested that the high pressure burrowing (associated with a higher rate of burrowing cycles) is an escape reaction.

Coelomic pressure measurements in *Leptosynapta* show that up to 20 cm. are generated during thrusts but pressures are only 1–2 cm. during tentacular scraping.

In *Golfingia* the maximum coelomic pressure of the burrowing cycle corresponds to penetration of the substrate; while in *Leptosynapta*, progression occurs between pressure peaks. *Golfingia* burrows by means of forward thrusts into the sand, *Leptosynapta* by lateral scraping. Although the two have vastly different maximum pressures (*Golfingia* at least ten times greater than *Leptosynapta*) in normal burrowing *Golfingia* probably utilizes a pressure only 2–3 times that of *Leptosynapta*.

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Organic content in developing squid eggs assessed from carbon, nitrogen, and ash, and its evolutionary significance. W. D. RUSSELL-HUNTER AND ROBERT J. AVOLIZI.

Eggs of cephalopod molluscs could be valuable to physiological ecologists, since they are relatively large but *not* cleidoic. Nearly 40 years ago, the considerable uptake of inorganic salts occurring during development of the eggs of the cuttlefish, *Sepia*, was demonstrated, but there has been little subsequent work. In the squid, *Loligo pealei*, Arnold's recently established normal stages (1–30) provide a chronology of development in spite of great variation with temperature. Determinations of wet and dry weights, of total organic carbon by "wet oxidation," of total nitrogen using an automatic micro-Dumas analyzer, and of inorganic "ash" were made on a series of eggs, dissected from their egg-strings.

The several strings of eggs laid at one time by a female squid contain hundreds of eggs remarkably uniform in organic content assessed as total nitrogen. Thus, many sequential determinations could be made on batches of closely comparable eggs. Differences between egg masses used, however, made it necessary to compute results in terms of a "standard" egg of modal ash-free dry weight. Mean values of total dry weight for individual "standard" eggs are 450 μg. (Arnold's stages 1–11), 500 μg. (15–20), 620 μg. (21), 750 μg. (24/25), and rise to 850 μg.–1.0 mg. by hatching (29/30). Mean wet weights are less consistent and reflect water uptake: 2.3–3.5 mg. (3–11), 4.5 mg. (15), 6.9 mg. (17), 15.2 mg. (24/25), and may rise to 23.6 mg. before hatching (29). Mean organic carbon values include 170 μg. (7), 188 μg.

(17/18), 181 µg. (25) and 191 µg. (29). Mean total nitrogen values are very consistent and include 40.6 µg. (3), 47.0 µg. (12), 46.8 µg. (14/15), 44.8 µg. (21) and 47.9 µg. (25). There is evidence of slight organic uptake from the egg-string material by the eggs. The dry weight increase results from changes in inorganic ash weights reflecting salt uptake particularly in the later stages: 50 µg. (1–11), 60 µg. (15), 120 µg. (17–19), 260 µg. (21), 380 µg. (24/25), and about 450 µg. (29 before hatching). After hatching the young squid retain only about 62% of their organic content (assessed as nitrogen) and a much smaller proportion of the egg's final inorganic salt content.

As in other molluscan examples, evolution of these eggs may have involved selection pressures to reduce the temporal extent of immature growth. However, since the squid egg is clearly not a closed box, full pre-adaptation for nonmarine colonization cannot arise.

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Observations on the fine structure of the fertilization membrane of Arbacia punctulata. Susumu Ito, Jean-Paul Revel and Daniel A. Goodenough.

During an examination of the fine structure of *Arbacia punctulata* eggs, we noted that the fertilization membrane displayed interesting ultrastructural characteristics not previously reported. The material was prefixed with acrolein purified according to the method of Aldridge; preparations made with other common fixatives showed similar features but less distinctly. A fully formed fertilization membrane is present 4 minutes after activation of the egg and persists to hatching. It is a dense structure about 500 Å thick which, in favorable sections, appears to be formed by an amorphous central region about 200 Å wide and two rows of circular profiles 150 Å in diameter. These circular images have been observed in either the outer or the inner layer but they have never been found in both in the same cross-sectional view of the fertilization membrane. This suggests to us that the circular profiles represent tubular structures oriented in different directions. When the circular profiles are not seen, the same layer appears to be a trilaminar structure formed by two dense lines each about 50 Å wide and separated by a space of 50 Å. Such images have been reported by others.

After insemination the vitelline envelope elevates from the oolemma as an amorphous layer. Within the first 4 minutes it progressively becomes organizd into the fertilization membrane described above. Early in this period the cortical granules are released and presumably contribute to the formation of this fertilization membrane. When dejellied, unfertilized eggs are treated with trypsin the thin, fuzzy external layer, presumed to be the vitelline envelope, is removed but the cortical granules appear unaffected by the enzyme. After fertilization the trypsin-treated eggs form an apparently normal hyaline layer but no fertilization membrane.

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Localization of tissue incompatibility and specificity of the overgrowth reaction in Hydractinia echinata. Frances Shapiro Ivker.

From previous work it has been established that tissue incompatibility between genetically varying strains is manifest by the induction of abnormal stolonic growth (overgrowth) in one of a pair of colonies grown together on a slide. In 10 strains studied, a hierarchy of overgrowth was established, correlated, but not absolutely, to the growth rates of the various strains. Colonies were stained with vital dye by feeding them *Artemia* stained with either Nile blue sulfate or neutral red. Two weeks later, colored endodermal cells were squeezed from the stolons, dissociated in Ca^{++}-Mg^{++}-free sea water at 19° C. for 30 minutes, then combined in pasteurized sea water where they reassociated, regardless of their origin from incompatible strains, leading to the hypothesis that the incompatibility factor is localized in the ectoderm. A possible mechanism, that could also account for Steinberg's binding energy hierarchy, would be a quantitative difference in the production of a surface-bound molecule, or a multiallelic qualitative difference, similar to seratypes in protozoa.

In nature, 1/200 of the shells collected display two distinct colonies with a barren zone between them, rather than the normal homogeneous single colony per shell. This may be the result of an overgrowth reaction in which both colonies contributed to the overgrowth mass.

Neither was able to destroy the other and the mass was invaded by algae, worms and other small marine life, leading to its eventual death and destruction. Algae inhibited further stolonic invasion of the area, producing a permanent zone of demarcation between the two strains.

The overgrowth reaction has, to this point, remained specific to contact between strains of *Hydractinia echinata.* Stolonic contact with the calyptoblastic hydroid *Campanularia,* the gymnoblastic hydroid *Bougainvillia,* human hair protein and agar has failed to induce overgrowth.

An intermediate form of incompatibility (no overgrowth, but a lack of fusion) has been observed between parent and offspring and half-siblings, again indicating a complex, multigenic control system.

Observations on the circulatory system of Magelona sp. MEREDITH L. JONES AND JOANNE V. SIMSON.

Examination of both living and sectioned specimens of *Magelona* sp., from well-sorted fine sand sediments of the Woods Hole area, revealed the presence of pulsatile sections in the dorsal blood vessel. In the anterior region of this polychaete (the first nine setigerous segments) there are two linearly-arranged chambers, each provided with a weak musculature, which receive blood from the posterior portion of the body and pass it anteriorly to a long, heavily muscularized part of the dorsal vessel; this, in turn, carries the blood to a pair of prostomial tentacles. These three vascular areas are set apart from one another by two valves which allow a precise and interrelated timing of contractions to send the blood anteriorly. Bypasses connect the two posterior chambers directly with an extensive ventral blood sinus; these function when the readily deciduous tentacles are lost.

Another type of vascular valve has been observed in the posterior dorsal vessel; these apparently facilitate the return of blood from the segmental vessels. These valves are segmentally arranged throughout the whole of the posterior region of the body of *Magelona* sp.

It has also been noted that the eversion of the proboscis of this worm is carried out by the hydraulic pressure of the blood, rather than of the coelomic fluid, as is the case in other polychaetes.

Pulsation rates of the anterior pumping mechanism are normally about 28–30 per minute; during anaesthesia with $MgCl_2$ they may slow to 24; and when exposed to the heat of a lamp, may speed up to 55.

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Ultrastructural observations on the development of cilia in the trochophore of Nereis limbata. VITAUTS KALNINS.

Cilia formation was studied in the prototrochal cells of the trochophore of the annelid *Nereis limbata* 8–12 hours after fertilization. The trochophores were fixed with glutaraldehyde, postfixed with OsO_4, embedded in Epon, and the differentiating ciliated cells in the prototroch were examined in the electron microscope.

During this period there is a progressive increase in the number of centrioles and cilia in the prototroch region. Typical immature centrioles or procentrioles are produced at specific sites on the mature centrioles. These sites are characterized by electron-dense plaques about 150 $m\mu$ in diameter and 40 $m\mu$ thick, which are attached by a somewhat less electron-dense material to the outside of the proximal part of the mature centrioles. The long axis of the procentriole is frequently but not always perpendicular to the long axis of the mature centriole. The close association of the procentrioles with the mature centrioles in the region of this electron-dense plaque suggests that the plaque may act as an organizing or initiating site for procentriole development.

The procentrioles increase in length, separate from the mother centriole, take up their position at the cell surface and become basal bodies by acquiring a cilium. As the cilia elongate they pass through channels present in the chorion, the distal ends of which are closed. The cilia must therefore penetrate through these closed ends in order to emerge. At the junction of ciliated cells, near the free surface, a desmosome and septate junctions are present.

This study was made in the Fertilization and Gamete Physiology Training Program (NIH grant T1 HD-26-06).

Colony fusion compatibility types in Botryllus schlosseri. STEPHEN KARAKASHIAN AND ROGER MILKMAN.

To gain basic information on colony fusion, in particular the number and nature of compatibility classes, several approaches were employed. To establish a minimum number of mutually incompatible classes, 5 colonies were made contiguous in all possible combinations. No heterologous fusions were observed. To estimate the effective number of compatibility types in the local population, random pairs of mature colony fragments were combined, with one heterologous fusion in 22 tries. Apposition of newly metamorphosed oözooids resulted in one fusion in 21 tries. Examination of carpets of *Botryllus* taken from nature revealed 78 fusions in 1262 contiguous borders. These data all suggest (simplest interpretation) that there are about 20 compatibility types in the Eel Pond population sampled. But the one-locus, multiple-allele model proposed by Oka and Watanabe and by Sabbadin on the basis of genetic crosses, in which colonies sharing at least one allele would fuse, requires about 80 alleles for fusion to occur at the frequency we have observed (roughly 5%).

The model also predicts invariable mother-daughter fusion (we have found exceptions); fusion of 87½% of sibling pairs in the progeny of a selfing (so far we find 1 out of 5); and fusion in 50% of pairs with a common mother only (we have seen 22 in 150). We have observed degenerative changes after some fusions, resulting in re-separation or in the disintegration of one or both partners, as has Sabbadin. The resolution of these interactions into several categories should lead to the definition of their genetic basis.

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Electron microscopic observations of gametes and early zygotes of Fucus and Palaemonetes. LAWRENCE D. KOEHLER.

Fertile fronds of *Fucus vesiculosus* were collected at Nobska Point, Woods Hole, Massachusetts. Gametes were obtained as described by Whitaker (1936). Specimens were fixed in a variety of fixative-buffer combinations. The best fixation was obtained at 4° C. in 2% glutaraldehyde, buffered to pH 7.2 with s-collidine, and containing 0.4 *M* NaCl. Cells were postfixed for 1 hour in cold osmium tetroxide, dehydrated, and embedded in Swiss Durcupan (Fluka).

Prepared tissue was sectioned at 600–900 Å with the LKB ultratome, stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963) and examined with the Philips 100 C electron microscope.

The cell body of the biflagellate *Fucus* antherozoid was 2–3 μ long with a large mitochondrial mass near the posterior end. It also had an irregularly shaped nucleus, and a chromatophore which contained lamella-like structures. The ova were between 50 and 90 μ in diameter. When they were released from the oogonial capsule they contained numerous large electron-dense granules distributed throughout the cytoplasm. Within a few minutes after release, most of these granules were located beneath the cell surface. The presence of a distinct cell wall was observed at 10 minutes subsequent to penetration by the antherozoid. The large granules were still present in the egg cytoplasm even after a thick cell wall had been formed and are clearly therefore not involved in its development.

Zygotes appeared to contain more Golgi structures than unfertilized ova. These Golgi structures may produce material involved in cell wall formation as demonstrated for higher plants (Linskens, 1966).

The non-motile spermatozoon of *Palaemonetes* was oval in shape (8–10 μ in long diameter) with a rigid spike (12 μ long) attached to one side. The spike was composed of densely packed filaments having regular cross-striations with a periodicity of 16/micron. A complex membranous structure was located near the base of the spike. The unfertilized eggs were 0.8 mm. in diameter and contained many large granules in the cortex. The spawned egg had a thick investment consisting of three distinct layers.

This study was made in the Fertilization and Gamete Physiology Training Program (NIH grant T1 HD-26-06).

Ultrastructural localization of acid phosphatase in rediae and cercariae of Cryptocotyle lingua. Paul L. Krupa, Arya K. Bal and Gilles H. Cousineau.

Acid phosphatase activity was sought with the aid of the electron microscope in the body wall of rediae and in the integument and secretory cells of cercariae of the trematode, *Cryptocotyle lingua.* Rediae, intramolluscan stages of developing cercariae, and free-swimming cercariae were fixed in cold cacodylate-buffered glutaraldehyde or in cold glutaraldehyde in Marine Molluscan Ringer at pH 7.4 with $CaCl_2$ added. Using β-glycerophosphate as substrate, some larvae were incubated for one hour at 37° C. in Barka and Anderson's modification of Gomori's medium at pH 5.0. Other larvae were incubated for 15 or 30 minutes at room temperature in Holt's version of Gomori's technique at pH 5.0 with cytidine monophosphate as substrate. Some experimental and control larvae were stained with ammonium sulfide and studied as wholemounts. All other specimens were postfixed in cold 1% osmium, dehydrated and embedded in Epon in the usual manner.

Wholemounts of experimentals showed lead sulfide precipitate at the surface of rediae and intraredial cercariae. In developing and free-swimming cercariae this precipitate also occurred in the penetration gland and penetration gland ducts.

At the fine structural level the acid phosphatase reaction product appeared as electron-dense granules and clumps of lead phosphate within the integumental folds of rediae and within the integument of cercariae. In some instances extremely dense concentrations of lead phosphate particles occurred in the cytoplasm near and around certain membrane-bound granules within the integument of cercariae. These granules may be lysosomes, even though for some unknown reason the reaction product appeared in their vicinity rather than within them.

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Postsynaptic effects of linearly increasing current in the presynaptic terminal of squid giant axon synapse. Kiyoshi Kusano.

The minimal presynaptic depolarization (MPD) for initiating the first detectable postsynaptic potential (PSP) and the correlation between the time courses of presynaptic depolarization and of the PSP were studied. Observations were made in normal preparations, in preparations perfused with tetrodotoxin (TTX), with tetraethylammonium ions (TEA) injected presynaptically, or with both. The presynaptic depolarizations were evoked by applying square current pulses or linearly increasing current pulses. In the synapse externally perfused with 10^{-6} g./ml. TTX, an MPD of about 25 mV. is required, as tested by square current pulses. Similar values were obtained from preparations in which TEA was electrophoretically injected in the presynaptic terminal and perfused externally with TTX. This level was, therefore, a little higher or about the same as the critical spike firing level at the normal presynaptic terminal where no PSP can be detected in the postsynaptic fiber before the presynaptic spike generation. By applying linearly increasing current pulses it was hoped to accomodate normal spike electrogenesis at the presynaptic terminal and to see the relation between the slowly increasing presynaptic depolarization and PSP. Most of the results obtained from normal synapses by this method did not induce accomodation of spike generation of the presynaptic terminal and firing of a presynaptic spike preceded the PSP. In some preparations, however, the critical firing level for the presynaptic spike increased to about 40 mV. A slowly rising PSP was demonstrated before the presynaptic spike occurred and the MPD was less than 30 mV. A similar slow rate of rise of PSP was seen upon application of linearly increasing currents to TEA-TTX treated synapses. The data thus provide additional evidence that Na-conductance and K-conductance increases during spike electrogenesis are not requirements for transmitter release. There is a correspondence of time courses of the PSP and the presynaptic depolarization, whether the latter is associated with a spike or with an applied current. The rate of rise of PSP appears to be primarily controlled by the rate and amount of presynaptic depolarization.

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Inactivation of motility in marine phyto-flagellates and invertebrate spermatozoa by phosphorothioate compounds. NORMAN LAZAROFF.

The toxicity of parathion to sensitive animal forms (insects) depends upon the conversion of parathion to an active oxygenated derivative (paraoxon). Current concepts of the mode of action of parathion hold that enzymes of susceptible tissues catalyze the conversion to paraoxon, which then functions as a potent acetylcholine esterase inhibitor. The toxic effects of paraoxon are approximated by a thioester isomer formed by heating pure parathion. Since acetylcholine esterase has been implicated in the functioning of sperm tails it was of interest to determine if parathion or its derivatives affected flagellar motility.

Dosage levels of 30 p.p.m. of chemically pure parathion had little or no effect on the motility of Chlorophyta (*Chlamydomonas, Platymonas*); Chrysophyta (*Dicrateria, Prymnesium, Monochrysis*); Euglenophyta (*Euglena, Eutreptia*); Phaeophyta (*Fucus* antherozoids); and invertebrate spermatozoa (*Arbacia, Spisula*). All of these systems were immobilized by comparable concentrations of heated parathion preparations (150° C. for 2 hours), containing the thioester isomer, although considerable differences in the time course of inactivation occurred. The chrysomonads as well as three unclassified algal flagellates (*Olisthodiscus*, "Nep." and "H.H." from R. R. L. Guillard) continue a slow regular flagellar contraction after progressive movement is largely inhibited. Four species of dinoflagellate genera tested (*Prorocentrum, Peridinium, Amphidinium,* and *Gymnodinium*) were immobilized by pure parathion as well as the heated preparation. Neither parathion nor the heated preparation inactivated motility of ciliated *Arbacia* larvae, from blastula to pluteus stages, at doses up to 100 p.p.m.

It was possible to reverse the immobilizing effect of heated parathion on *Prymnesium* and *Spisula* spermatozoa by washing after treatment.

The data support the idea that a phosphorothioate-sensitive esterase mechanism functions in close relationship with the flagellar apparatus in the systems studied.

This study was made in the Fertilization and Gamete Physiology Training Program (NIH grant T1 HD-26-06).

The formation of insoluble protein in the ocular lens. SIDNEY LERMAN, MARSHA HOLLANDER AND SEYMOUR ZIGMAN.

The insoluble protein fraction of the lens increases progressively with age until it represents more than 50% of the total lens proteins. The amino acid composition of rat and dogfish lens insoluble protein is very similar to that of gamma crystallin (one of three soluble lens proteins) and both of these protein fractions shown E^1_1 values of 18 as compared with 8 for alpha crystallin. The high E^1_1 values are due to the relatively high tyrosine content in these proteins which also causes the hyperchromicity. Further evidence regarding the similarity of gamma crystallin and the insoluble protein fraction is demonstrated by C^{14} amino acid incorporation experiments (employing C^{14} leucine or C^{14} aspartic acid) in which the rates of incorporation of these amino acids over a 24-hour time period were almost identical for those two protein fractions, while alpha crystallin had a much higher rate. The SH and S-S content of old gamma crystallin is identical with that of the insoluble protein, providing further evidence of the close relationship between these two lens proteins.

The tyrosine residues in gamma crystallin show an unusual ionization behavior (with an ionization constant of 11 as compared with a constant of 10 for tyrosine alone) and gamma crystallin also has an abnormal absorptivity (hyperchromicity) as well as being the only lens protein which has an appreciable ESR signal while all the other protein fractions must first be irradiated in order to demonstrate the presence of free radicals. Preliminary studies indicate that bityrosine is present in small quantities in old gamma crystallin and in the insoluble protein fraction. The presence of this compound would explain the unusual ionization behavior and the hyperchromicity observed in gamma crystallin. It could also serve as an intra- or intermolecular covalent cross-link in the formation of the insoluble protein which appears to derive mainly from gamma crystallin in the rat and dogfish lens. Aside from the foregoing, the increase in S-S bonds in old gamma crystallin and in the insoluble protein indicates that both S-S bonds and bityrosine play an important role in the formation of the insoluble lens protein. It is also possible that bityrosine may represent the fluorescent compound that has been noted in a variety of lenses and which increases in concentration as the lens ages.

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Observations on feeding in Magelona sp. ROBERT F. McMAHON AND MEREDITH L. JONES.

Observations were made on *Magelona* sp., a polychaete collected at Stony Beach, Woods Hole, Massachusetts. The animals were observed in a sand chamber between two glass plates.

Magelona constructs a vertical burrow open at the sand-water interface. Two prostomial tentacles, originating laterally and posteriorly to the mouth, are extended from the burrow opening. These tentacles are up to 15 mm. in length, ¼ the worm's total length.

The distal ⅘ of each tentacle is equipped with four rows of small adhesive papillae. There are several large cells at the base of each papilla which appear to secret the mucus which renders the papillae adhesive.

Food particles, *i.e.,* clumps of diatoms, algae, or small animals, adhere to the papillae upon contact. After contact, a proximal portion of the tentacle loops about the particle. The distal papillae then release the particle while those of the proximal end of the loop attach to it. The loop is straightened, and the particle has progressed down the tentacle. Loop formation is repeated carrying the particle down the tentacle into the burrow.

Since the proximal ⅕ of the tentacle lacks papillae, at this point loop formation ceases. The particle is released by the papillae and enters the space between the tentacles where it is carried to the mouth. There are no cilia or feeding currents on the tentacles or prostomium for particle transport. Instead a mucus thread appears to be produced in this region to carry food to the mouth.

Once ingestion occurs the food moves rapidly through the gut of the first nine setigers to a glandular region between setigers 10 and 25. Digestion and assimilation appear to take place here as the more posterior regions of the gut contain only fecal material.

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The effects of hyperbaric oxygen on cell movements, differentiation and growth during regeneration in Tubularia. JAMES A. MILLER, JR., FAITH S. MILLER, DAVID L. DESHA AND BERNARD ROBINOWITZ.

Oxygen at pressures above 2 atmospheres blocks succinic dehydrogenase activity and differentiation in *Tubularia* embryos (Miller *et al.*, 1966). When tested on regenerating 10-mm. stems, it increased length of regenerates at pressures of 1, 1½, 2, and 2½ atmospheres absolute (A.A.). At 3 A.A. and higher regeneration was partially or completely blocked. This is not a direct effect of pressure since exposures to 2 and 3 A.A. of 10% O_2 + 90% N_2 failed to cause blockage. Blockage requires about 24 hours to develop and is completely reversible for as long as 5 days. Stems exposed for 24 hours to 3 A.A. regenerated longer hydranths than did controls. Longer exposures resulted in complete blockage of differentiation in some experiments. When stems were placed in 3 A.A. after emergence of the hydranths there was no effect on growth of the stem for 12 hours; however, between 12 and 36 hours the increase in length of the experimental stems was only ⅛ that of the controls. Thus, hyperbaric oxygen produces greater effects on differentiation and growth than upon the first stages of regeneration which involve cell movements.

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Detrimental effects of linear alkylate sulfonate on larvae of selected marine invertebrates. D. F. MOFFETT AND D. S. GROSCH.

Developing embryos of *Arbacia, Asterias, Spisula, Chaetopterus,* and *Molgula* were exposed to concentrations of biodegradable LAS ranging from 0.2 to 5.0 p.p.m. in sea water beginning at 15 minutes post-fertilization and continuing throughout the period of development. Concentrations in some rivers and estuaries fall in this range and sewage effluent exceeds it. The U.S. Public Health Service drinking water standard recommends a limit of 0.5 p.p.m. Gross developmental abnormalities resulted in all of the genera studied. The following designations in p.p.m. taken from the plotted dose-effect curves are for 50% and 100% abnormal, respectively: *Arbacia* 0.9, 1.0; *Asterias* 0.7, 3.0; *Chaetopterus* 1.1, 1.5; *Molgula* 1.6, 2.0; *Spisula* 2.0, 3.0. Steep slopes on some of the curves suggest a tolerance level phenomenon which may be obscured in other cases by heterogeneity of gametes obtained surgically. The degree of morphological abnormality increased with increasing concentration above that giving 100% abnormal embryos. In many instances a cell-wide loss of pigmentation, reminiscent of Heilbrunn's "surface precipitation reaction," was observed. Detergent had a slight parthenogenetic effect at 10 p.p.m. for one hour, scored on the basis of fertilization membranes, but most of the eggs were cytolyzed.

Gametes of *Arbacia* were immersed in a dilution series from 10.0 to 1.0 p.p.m. for periods up to one hour, washed and used in fertilization experiments with untreated gametes. Results from these experiments and from studies of early cleavage in detergent indicate that at concentrations used, LAS can damage unfertilized eggs but not sperm. Furthermore there was no decrease in percentages of eggs showing fertilization membranes and cleavage was not delayed. Evidently the mitotic apparatus was not damaged.

Detergent used in these studies was an interim reference sample provided by the Soap and Detergent Association analyzed as LAS 60.8%, Na_2SO_4 36.1%, free oil 0.4% and H_2O 2.7%.

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Effect of some organic cations on generator potential of stretch receptor of crayfish. SHOSAKU OBARA.

In both slowly and rapidly adapting stretch receptors of crayfish the generator potential can still be elicited by mechanical stimuli, after all the Na^+ of the bathing medium is replaced with various organic cations. In the presence of tris(hydroxymethyl)aminomethane, Tris, the generator potential is particularly large, about 30 to 50% of that in the control saline. Persistence of the generator potential in the modified salines is not due to retention of Na^+, the possibility of a diffusion barrier being eliminated by four types of experiments. Ionic contributions to the generator potential by Cl^- and Ca^{++} can also be excluded. Contribution of K^+ is suggested by measurements of the generator potential equilibrium value, which lies below zero level in the normal saline, and shifts to a more negative level in Tris saline. In the presence of various monovalent cations replacing Na^+, the relative amplitude of generator potentials is as follows: $Na^+ > Li^+ > \text{Tris} > \text{TMA} > \text{choline} \geqq \text{TEA}$, and probably indicates the order of the permeability of these cations in the active generator membrane. Except for the first two, these cations are completely inert for the spike electrogenesis of stretch receptor neuron under the present conditions. Thus, while the electrogenesis of the generator membrane must be due to an increased permeability for monovalent cations, the active generator membrane appears to be less selective for different cation species than is the receptive component of other receptors, or the conductile component of the stretch receptor neuron.

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Entry of Cerebratulus spermatozoa into Echinarachnius eggs. PATRICIA J. OLDS AND C. R. AUSTIN.

An attempt was made to confirm and extend the observation of Robert Chambers (1933) that the spermatozoa of the nemertine *Cerebratulus* are capable of penetrating sand dollar (*Echinarachnius*) eggs. With both living and fixed and stained material, observed by phase-contrast microscopy, it proved possible to demonstrate that several entire spermatozoa did enter many of the eggs seminated. The penetrating spermatozoa remained motile in the egg cytoplasm for several hours and the stained sperm heads within the egg were unchanged in appearance, resembling those outside. The spermatozoa appeared to enter immature crab, *Cancer,* eggs in a similar manner, and at least partially to penetrate the membrane of *Arbacia* eggs. Preliminary studies by electron microscopy further corroborate Chamber's report of entry of *Cerebratulus* spermatozoa into *Echinarachnius* eggs. The spermatozoa appeared to retain their plasma membrane and to lie free in the egg cytoplasm; there was no evidence of an enclosing vesicle as described by Chambers.

The *Cerebratulus* sperm head was extremely long, tapering gradually to a fine, but blunt, tip. In the act of penetrating homologous eggs, the spermatozoa exhibited a very vigorous rotating or oscillating motion as they passed through the chorion and plasma membrane. Observations of sperm ultrastructure indicated that the nucleus accounted for most, if not all, of the head. The mitochondrial body was asymmetrical and extended anteriorly around the nucleus for a short distance. No structure positively identifiable as an acrosome was seen, but a small body, possibly an acrosome, occupied the tip of the sperm head. *Cerebratulus* spermatozoa showed no obvious acrosome reaction in the fertilization of homologous eggs, as judged by lack of any observable change during or after penetration of the chorion.

From these observations, it appears probable that *Cerebratulus* spermatozoa gain entry to foreign eggs without undergoing an acrosome reaction and consequently without the sperm-egg membrane fusion which is known as a normal fertilization process in several marine species.

This study was made in the Fertilization and Gamete Physiology Training Program (NIH grant T1 HD-26-06).

Additional information concerning Tiedemann's bodies of Asterias forbesi (Desor).

S. M. Paracer.

Tiedemann's bodies have been imperfectly described. Each gland is covered externally by peritoneum and is composed of branched follicles that open into a central lumen which is connected to the circumoral water vascular ring canal by a short duct. Each follicle is lined by an epithelium composed of tall ciliated cells and is embedded in loose connective tissue. The function of Tiedemann's bodies is a matter of dispute. Impressive quantities of secretion are produced and enter the water vascular system, a fact not hitherto reported. The origin, nature, and function of the secretion are unknown. It was suspected that the glands might show a reaction when the starfish is injured and owing to the incursion of foreign matter. To test the latter notion, India ink, coelomic fluid from another individual of the same species, sperm of *Arbacia punctulata,* and *Uronema* cultured with bacteria, were injected into the perivisceral coelom of the experimental starfishes. The glands responded by conspicuous swelling, due to follicular dilation and the discharge of the follicular epithelium. None of the substances introduced into the test animals entered the Tiedemann's bodies. The cells lining the follicles may be discharged singly or *en masse* and replaced by activity of the epithelial lining. The effect was maximal 18 hours after introduction of the foreign matter into the perivisceral coelom. The follicles may be dilated, but empty, suggesting that the discharged cells are evacuated from the gland into the water vascular system. This could be due to contraction of the muscle fibers in the gland. The ultimate fate of the discharged cells is unknown. To discover whether depriving the animal of coelomocytes exerts an effect on the gland, coelomic fluid was drained away. Its replacement was accompanied by marked shrinkage of the glands and depletion of their cellular contents.

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Nucleic acid and protein synthesis in dogfish peripheral blood in vivo and in vitro.

Thoru Pederson and Seymour Gelfant.

As a result of our previous work on nucleic acid synthesis in tissue cultures of dogfish peripheral white blood cells, we have become interested in studying the *in vivo* situation. In addition to describing synthesis *in vivo,* we hope to use the *in vivo* data to better understand our *in vitro* system.

We have used the smooth dogfish, *Mustelus canis,* and have been concerned with two white blood cells of the peripheral circulation, hemocytoblasts and lymphocytes. Our analyses have been performed by biochemical, cytophotometric and radioautographic techniques.

Cytophotometric measurements of Feulgen-stained nuclei showed that 18% of the hemocytoblasts and 3% of the lymphocytes were engaged in DNA synthesis *in vivo*. This synthesis was also measured by the incorporation of H^3-TdR into acid-insoluble material (around 300 cpm/10^6 cells/5 min.). When peripheral white blood cells were placed in culture, DNA synthesis was abruptly reduced, but there followed a wave of activity from 6 to 24 hours *in vitro*. After 24 hours, synthesis continued at a reduced rate throughout 21 days *in vitro*. This pattern was observed by radioisotope incorporation, cytophotometry and radioautography, and the latter two techniques revealed that, as *in vivo*, hemocytoblasts and lymphocytes were the cells responsible for the activity.

RNA synthesis has been measured, biochemically and by radioautography, by the incorporation of H^3-CR *in vivo* and *in vitro*. *In vivo*, the rate of RNA synthesis was around 1600 cpm/10^6 cells/hour, whereas *in vitro* synthesis fell immediately to about 20% of the *in vivo* rate and continued at that level throughout 21 days. Radioautographs have defined hemocytoblasts and lymphocytes as the basis of this incorporation. Protein synthesis, as measured by the incorporation of H^3-leucine into TCA-precipitable material, occurred at equal rates *in vivo* and after 21 days *in vitro* (about 850 cpm/10^6 cells/hour).

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The control of skeleton differentiation in sea urchin embryos. A molecular approach. RICHARD PELTZ AND GIOVANNI GIUDICE.

Triradiate skeletal spicules are first observed at the completion of gastrulation in sea urchins. The formation of spicules involves deposition of calcium carbonate in an organic matrix. Nakano *et al.* (1963) demonstrated a great increase in calcium uptake at the mesenchyme blastula stage. Giudice (1967) initially observed that Actinomycin D administered at the low dosage of 2.5 μg./ml. selectively inhibits skeleton formation in *Paracentrotus lividus*. Our present experiments with *Arbacia punctulata* indicate that embryos developed in the presence of 0.5 μg./ml. Actinomycin D from the swimming blastula stage onward will not develop skeleton, but will exhibit morphology which is normal in other respects. Arm elongation dependent on skeleton formation does not occur; however, the embryos do form pigment cells and a stomodaeum, and show all the other morphological characteristics normally acquired after spicule formation. Measurement of Ca^{45} uptake showed an inhibition of calcium uptake into embryos treated with Actinomycin D (0.5 μg./ml.) as well as into the 800 *g* supernatant which does not contain spicules. Non-treated embryos exhibited an increase in calcium accumulation both in the total homogenate and 800 *g* supernatant, commencing at the mesenchyme blastula stage. Calcium accumulation in all cell fractions was observed after fractionation of non-treated embryos.

In order to determine whether the actinomycin is acting at the gene level, embryos were pulsed with H^3-uridine for 45 minutes at the late mesenchyme blastula stage. RNA was extracted with SDS-phenol at 60° C. Sucrose gradient analysis indicated a consistent inhibition of total RNA synthesis in several experiments. The inhibition seems to be greatest in the region of the gradient lighter than 18S. Preliminary experiments do not demonstrate any major difference in protein synthesis between Actinomycin-treated and control embryos at different stages of skeletal development through the pluteus stage.

In conclusion, our experiments demonstrate that the increase of calcium uptake, concomitant with skeleton formation, is inhibited by low doses of actinomycin. Our results are consistent with the hypothesis that this inhibition is exerted through the specific inhibition of the synthesis of some particular class(es) of RNA.

This study was made in the Fertilization and Gamete Physiology Training Program (NIH grant T1 HD-26-06).

Effect of organic solvents on the melanin-dispersing activity of eyestalks from the fiddler crab, Uca pugilator. K. RANGA RAO, CLELMER K. BARTELL AND MILTON FINGERMAN.

Eyestalks of the fiddler crab, *Uca pugilator,* were extracted in 13 organic solvents. The soluble material, after the organic solvent had evaporated, was suspended in sufficient sea water to give a final concentration of one eyestalk complement per 0.05 milliliter. Melanin-dispersing activity was determined using eyestalkless *Uca pugilator*. No overall relationship between the melanin-dispersing activity of the extracts and the polarity of the organic solvents was detected. However, when the data for the alcohol series alone were considered, it was clear that the melanin-dispersing activity of the alcohol-soluble fraction increased with increase in the polarity of the alcohol used. The melanin-dispersing activity of the alcohol extracts was in the following order of increasing potency: amyl alcohol < butanol < propanol < ethanol < methanol. The eyestalk material soluble in amyl alcohol, isoamyl alcohol, ether, isopropyl ether, and acetone had only traces of melanin-dispersing activity. The melanin-dispersing activity of the methanol-soluble fraction of the fresh eyestalks was four times more active than the sea-water extract of fresh eyestalks while the ethanol-soluble fraction was three times more active than a sea-water extract. The high melanin-dispersing activity of the ethanol-soluble fraction of the eyestalk was demonstrable only when freshly dissected eyestalks were used. Freeze-drying, oven-drying, or pretreatment of fresh eyestalks with acetone, ether, isopropyl ether, chloroform, or petroleum ether resulted in a marked decrease in the melanin-dispersing potency of the ethanol-soluble fraction, whereas the melanin-dispersing activity of the ethanol-insoluble fraction was unaltered and remained similar to the activity of the eyestalk extracts prepared directly in sea water.

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Some observations on the fine structure of septate junctions. JEAN-PAUL REVEL.

The term septate desmosome was introduced by Wood in 1959 to describe an intercellular junction, now known to be peculiar to invertebrates, in which a series of parallel lamellae bridge the intercellular space between the plasmalemmas of two neighboring cells. Wood believed that the septa had the structure of unit membranes. More recently Locke suggested that the septa were in fact continuous with the plasma membrane. In tangential sections through the septate desmosomes of an insect, he found a hexagonal pattern which he believed represented the septa. He suggested, therefore, that the septa were not "shelves" but rather formed a series of hexagonally shaped compartments, each akin to a lipid micelle. We have now examined the septate junctions in a mollusk and several echinoderms, using aldehyde fixation followed by OsO_4 and uranyl acetate staining *en bloc*. While the typical "unit membrane" structure was clearly resolved in all plasma membranes, the septa always showed a finely fibrillar structure, rather than the trilaminar appearance expected if they had the same molecular organization as the plasma membrane. The outer leaflet of the plasmalemma could be seen as a continuous layer distinct from the septa. In tangential sections through septate desmosomes of *Arbacia punctulata* and *Asterias forbesi* parallel linear structures were observed, as described by Wood, suggesting that the septa were indeed shelf-like structures. Similar images were obtained in *Mya arenaria* but, here, a hexagonal pattern similar to that described by Locke was present in the same tangential sections. Thus, the hexagonal pattern does not necessarily represent the configuration of the septa. The hexagonal pattern is different from that seen in several electrotonic junctions in vertebrates. In the latter case there is a hexagonal packing of solid bodies, while in the septate junctions the hexagonal pattern is one of holes in a substrate.

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Studies of radiation effects and lifetime characteristics of the light output of scintillation liquid light standards. G. T. REYNOLDS, A. LIUZZI, J. W. HASTINGS, B. L. STRUNK AND T. REYNOLDS.

The importance of calibrated liquid light sources in quantitative studies of bioluminescent and chemiluminescent reactions has been pointed out by Hastings and Weber (1963). Results have been published, obtained by independent methods, for the light output of a standard consisting of a long-lived radioactive substance (hexadecane-1-C^{14}) in a non-volatile solvent and scintillator (three grams of PPO and 50 mg. of POPOP per liter of mono-isopropyl biphenyl). Recent measurements, reported here, indicate that the light outputs of unsealed standard solutions change with time, and that in some solutions prepared and sealed several years ago, the light output has either decreased, or the original output cited by Hastings was too large.

Unsealed light standards have been tested for their scintillation efficiency using Cs^{134} gamma rays, and found to be less efficient than unloaded scintillators. High radiation exposures to Cs^{137} gamma rays do not cause significant decreases in the scintillation efficiency.

The light standards are subject to the continuous radiation of the relatively low energy decay electrons of C^{14} (average energy of 50 kv.) and, to give out light, must respond to these low energies. It is known that the organic scintillators become non-linear in their response to low energy particles, so that more significant tests remain to be made subjecting the scintillators to low energy electron beams. Consideration should be given to the fact that the distribution of ionization relative to the organic molecules will be different for internal C^{14} radiation and external radiation. The disintegration of C^{14} leads to the production of N^{14} with some recoil energy. In view of the relatively low magnitude of dissociation energies for liquid scintillator components, this may also bear upon the observed degradation, as may the quenching effect of N^{14}.

For reliability these sources should be made up just prior to use.

This research was supported by AEC contract AT (30-1)-3406.

Scintillomicroscopy utilizing image intensification techniques for the detection of radioactive tracers. G. T. REYNOLDS, B. L. STRUNK AND A. W. SENFT.

It has been pointed out (Reynolds, 1963) that an image intensifier can detect radioactivity in biological systems viewed at high magnification. Due to the high sensitivity of image intensification systems, this method offers advantages over conventional autoradiography in certain cases.

The basis of the method is the detection by the image intensifier of the light emitted when a radioactive decay product strikes a thin scintillator placed directly over the specimen—thus the term "scintillomicroscopy." The image intensifier provides sufficient gain so that each electron leaving the cathode results in many developed grains in the camera film photographing the output phosphor. Thus the limiting statistics occur at the input cathode. The scintillator must be thin enough to preserve some spatial resolution (usually limited to the order of 20 microns) but thick enough to produce several hundred photons in response to the excitation of the decay product. The method has been applied to C^{14} detection at 1 × magnification, by lens coupling and by means of fiber optics input windows in the image intensifier. It has also been applied at magnifications 4 ×, 10 ×, and 22 ×.

Sufficient experience has been gained to permit a quantitative discussion of the method, providing criteria for assessing its value in particular applications, compared to conventional autoradiographic techniques, which are limited to 1 × magnification, require much longer exposures, but provide better spatial resolution. The problem is to provide sufficient signal by means of the light from the scintillator to overcome the noise of the intensifier system due to the thermal electrons leaving the cathode.

An equation relating signal and noise has been derived and tested experimentally, that can be used to determine the time of exposure required if the radiation dose must be limited, or the radiation dose required if the exposure time is limited.

This research was supported by AEC contract AT (30-1)-3406 and by NSF grant GB 5468.

Studies on the synthesis of ribosomes in sea urchin embryos. HARRY ROY AND GIOVANNI GIUDICE.

In several organisms it is believed that ribosomal subunits are made in the nucleus and transmitted to the cytoplasm as 60S and 40S particles. It was desired to see if such precursor subunits could be detected in sea urchins. A method for distinguishing nuclear and cytoplasmic material was developed: plutei or gastrulae were disaggregated by the method of Giudice, and the resulting free cells were homogenized in 0.01 M Tris, 0.025 M KCl, 0.0004 M Mg-acetate, pH 7.4. The homogenate was made 0.25 M in sucrose and centrifuged at 600 g for 10 minutes. The homogenization and centrifugation were repeated twice to give an enriched nuclear preparation, as judged by phase microscopy and by electron microscopy (courtesy of Dr. Gertrude Hinsch). The nuclei were lysed in a Frence press. The 10,000 g supernatant of this lysate was analyzed on a sucrose gradient for the presence of particles sedimenting at between 100S and 20S. Cytoplasmic ribosomes derived from post-mitochondrial supernatants were run in parallel gradients.

Experiments were done by adding H^3-uridine (0.27 μC./ml., 24.7 C./mM) to cultures of plutei (5000/ml., in the presence of antibiotics), and sampling at one and three hours thereafter. Within one hour of the addition of label, the nuclear radioactivity was high and sediments heterogeneously, with shoulders at 65S and 30S. The cytoplasmic activity is lower, with little peaks in the 60S and 40S regions. Two hours later the nuclear label is considerably reduced, while in the cytoplasm the specific activity has increased and sediments in distinct components of 80S, 60S, and 40S. The two slower components sediment in the manner characteristic of animal ribosomal subunits. In addition, preliminary experiments indicate that the 60S particles contain 28S RNA. The specific activity of the ribosomal subunits is higher than that of the whole ribosomes at one hour; at three hours, however, the ribosomes are more intensely labeled than the subribosomal particles. Although more experiments are needed to support a definitive conclusion, these results are consistent with the hypothesis that in the sea urchin, ribosomes are synthesized in the nucleus in the form of 60S and 40S particles which become assembled into 80S ribosomes in the cytoplasm.

This study was made in the Fertilization and Gamete Physiology Training Program (NIH grant T1 HD-26-06).

The effect of dinitrophenol and other phosphorylation uncouplers on the mitotic apparatus (MA) of marine eggs. NOBUAKI SAWADA.

The effects of various phosphorylation uncouplers on the mitotic apparatus of marine eggs were observed under the polarizing microscope. In the egg of *Pectinaria gouldi,* 2,4-dinitrophenol does not inhibit breakdown of the germinal vesicle but blocks MA formation at a concentration of $3 \times 10^{-4} M$ in sea water. It also causes the formed MA to lose its birefringence within two minutes of application. These effects are reversible and birefringence reappears completely within 10 minutes after washing with sea water. 4,6-Dinitrocresol, pentachlorophenol and carbonyl cyanide-p-trichloromethoxyphenyl hydrazone also give similar effects at the concentrations of $3 \times 10^{-4} M$, $\frac{1}{4}$ saturation ($5 \times 10^{-5} M$) and $5 \times 10^{-6} M$, respectively. One of the nitrophenols, o-nitrophenol, which has no effect on mitosis and metabolism, also does not affect the MA.

NaN_3 gives similar effects at $5 \times 10^{-3} M$ but acts more slowly than the above reagents. Na-arsenite at $5 \times 10^{-3} M$ also causes the MA to disappear but the effect occurs very slowly (30 minutes) and is irreversible.

In the eggs of *Spisula solidissima* the effects of these reagents on the first meiotic MA are the same as in *Pectinaria* eggs. In sea urchin eggs, *Lytechinus pictus,* they inhibit MA formation, but do not disperse the already formed MA.

DNP gives no effects on the MA of *Pectinaria* isolated with hexylene glycol. These results suggest that oxidative phosphorylation has an important role in the formation and maintenance of the MA.

Supported by grants to L. I. Rebhun from the American Cancer Society and the National Science Foundation.

Attempts to demonstrate persistent sperm plasma membrane following gamete fusion in Arbacia oocytes utilizing fluorescent antibodies. LEONARD M. SCHECHTMAN AND CHARLES B. METZ.

This study attempted to determine if sperm plasma membrane persists following sperm-oocyte membrane fusion. *Arbacia* oocytes were selected because insemination results in polyspermy and because a readily visible, persistent sperm-induced protrusion remains at the site of sperm-oocyte interaction (Franklin, 1965). Fluorescein-conjugated anti-sperm sera were employed in an attempt to specifically label any sperm membrane component of such "blebs." Glutaraldehyde (5% in sea water, pH 6.1, 1:1 with sample) preserved blebs and failed to inhibit reaction between sperm surface antigens and antibodies prepared against whole sperm in rabbits.

Untreated sperm and eggs displayed a low degree of autofluorescence. Sperm labelled with fluorescein-conjugated anti-*Arbacia* sperm antibody fluoresced over their entire surfaces. Pretreatment of oocytes with the same antibody resulted in a strong emission from their surfaces. Fluorescein-conjugated control rabbit globulin failed to cause fluorescence of oocytes or sperm.

Apparently oocytes possess antigen(s) in common with sperm. Confirmation of such common antigens came from Ouchterlony agar diffusion tests, where precipitation was observed between egg homogenate and anti-sperm antibody.

Attempts to eliminate cross-reaction between egg and anti-sperm involved repeated absorptions with excess mature unfertilized whole eggs. Whole egg absorbed antibody treatment resulted in fluorescence of sperm, oocyte, and oocyte bleb surfaces. Possibly such absorption is incomplete and further treatment of the antibody preparation might produce a reagent with the necessary resolution.

This study was made in the Fertilization and Gamete Physiology Training Program (NIH grant T1 HD-26-06).

A study of the kinetics of cellular proliferation of the Krebs-2 transplantable solid carcinoma and co-existing duodenum as determined by ^{3}H-T uptake and autoradiography. SISTER ROSARII SCHMEER, O.P.

An anticancer principle called mercenene, extracted from the clam *Mercenaria,* causes oncolysis of the Krebs-2 (K-2) carcinoma in CF1 mice. Cytological light and electron microscopy investigations of tumors from treated animals have failed to reveal the agent's mode of anticancer activity. In attempting to analyze the mechanism whereby mercenene is affecting the death of cancer cells, and to observe the effect of the extract upon the turnover time of co-existing duodenal tissue, the cellular kinetics of untreated K-2 carcinoma and duodenum were determined by ^{3}H-T uptake and autoradiography. In describing the sequence of events from one mitotic division to the next mitotic division a total of 184 mice with a palpable four-day-old tumor was used. Each animal received subcutaneously, at time zero, 0.80 μC./gm. body weight of triated thymidine. The mice were serially sacrificed, three per time interval, from 15 minutes to 24 hours after ^{3}H-T administration. Autoradiographs of tumor and duodenum sections were prepared and developed after two weeks. The mean duration of various phases of the cell cycle is based on mitotic curves derived from the rate of appearance and disappearance of labeled mitoses. For the K-2 carcinoma the mean S period (DNA synthetic time) was 7.5 hours, G_2 (post-DNA synthesis) 2 hours, G_1 (pre-DNA synthesis) zero, M (mitosis) 2.5 hours, and generation time 12.0 hours. The co-existing duodenum had a cell cycle essentially similar to that of the duodenum of non-tumor bearing mice. The S period was 7.5 hours, G_2 equalled 1 hour, G_1 was 7.5 hours, M was 1.5 hours, and the generation time 15.5 hours. The presence of the tumor does not seem to influence the turnover rate of the "normal" duodenal population. An investigation has been initiated to determine what effect mercenene may have on the normal cell cycle of the K-2 and co-existing duodenum. These studies may reveal the agent's mode of neoplastic effectiveness and oncolytic activity.

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Six fundamental problems in the Phylum Ectoprocta. THOMAS J. M. SCHOPF.

Despite a literature of 3600 papers directly concerned with ectoprocts, and a very diverse fauna of recent species (approximately 4000) and fossil species (approximately 15,000), several fundamental problems of ectoprocts remain unsolved. Six of these seem especially crucial to me.

1. How does the calcareous skeleton develop and what are the homologous skeletal wall layers, especially in families of the Orders Cheilostomata and Cyclostomata?

2. What are the factors controlling the formation of ectoproct polymorphic structures in general, and avicularia in particular? To what extent is the occurrence of any individual avicularium the result of genetic or environmental conditions?

3. What sort of biochemical control is responsible for the following cycle: formation of feeding polypides, then formation of an embryo and larva, followed by degeneration of the feeding polypide into a brown body, or compact pigmented structure? After release of the larva, a new feeding polypide forms from tissue lining the body wall. This cycle may be repeated more than 4 times within a single skeleton, with the accumulation of 4 or more brown bodies.

4. What are the excretory compounds? How are these related to the food (plankton) of the polypide?

5. What is the filtration rate of this phylum which is entirely composed of filter feeders? How is this related to oxygen utilization?

6. What is the form, function and biological role of the chief morphological features used in determining relationships among ectoproct higher taxa? What are the evolutionary trends in these features? How well do lineages suggested by these analyses support or conflict with one another? And what are the implications of this phylogenetic analysis on the present classification?

These basic biological questions of the Ectoprocta have yet to be answered.

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Summary of rediscovered type collections of North American east coast Ectoprocta. THOMAS J. M. SCHOPF.

North American east coast and European west coast ectoproct (bryozoa) faunas appear to differ from each other considerably more than earlier workers realized. Accordingly the American species names (several long considered synonyms of European forms) may be valid and hence careful biological and nomenclatorial attention must be paid to these type collections. Chief among these collections are those described by Stimpson (11 species, 1853), Packard (12 species, 1863–1867), Verrill (15 species, 1872–1901), Smitt (33 species, 1872–1873), and smaller lots described by Desor (3 species, 1848), Leidy (4 species, 1855), Dawson (4 species, 1859–1865), and Pourtales (7 species, 1867).

Curation of collections of various museums in the past 3 years yielded the following collections, several of which had long been considered "lost": 8 + of Packard's types are at the Museum of Comparative Zoology (MCZ), Harvard; 11 of Verrill's types are at the Peabody Museum, Yale, or the U. S. National Museum; 18 lots of probable syntypes of Smitt (whose extant material had long been thought to be only at Stockholm) were located at the MCZ, as were 3 types of Pourtales; 1 type of Desor and 3 of Dawson are at the Peabody Museum. Collections of Stimpson and Leidy were not found although search was also made at the Philadelphia Academy of Sciences.

Dr. Frank J. S. Maturo, Jr., collaborated in searching for and identifying material of Verrill, Dawson and Desor, and Dr. Joseph Hazel assisted in locating Smitt's collection.

Supported by a grant from the Ford Foundation to the MBL Systematics-Ecology Program.

Comparison of observation of C^{14} L-proline uptake in Schistosoma mansoni by means of scintillomicroscopy and conventional radioautography. ALFRED W. SENFT, BRIAN L. STRUNK, GEORGE T. REYNOLDS AND CYNTHIA S. GATES.

The principal finding shown by the intensification apparatus was that the male becomes much more radioactive than the female. Areas of special concentration were noted in the cephalad aspect of the male both anterior and posterior to the ventral sucker. The female, although generally considered to be metabolically more active than the male, showed considerably less uptake. There was a notable paucity of deposit in the vitellaria but some accumulation in the anterior third of the soma. When comparison cryostat sections were radioautographed, areas of intensity were confirmed and localized.

In the male: There is intense anterior localization which involves the distal esophagus and may include the neural ganglionic ring. The six to eight testes become moderately tagged. Sperm was not found to be significantly radioactive, although the short incubation period might have limited proline incorporation at this site. The stellate nuclei of the subintegumentary stromal zone become markedly radioactive, as does the undulating surface of the integument. Heavy deposits within segments of the cellular layer of the male gut were noted.

In the female: There is generally much less proline uptake in the integument when compared to the male. Relatively intense concentration in that portion of the oviduct containing maturing ova was seen. Satisfactory cross-sections of an egg *in utero*, or of the Mehlis gland were not achieved; thus it was not possible to determine whether the eggs become tagged as the shell is formed. The vitellaria and vitelline ducts were never especially radioactive, nor was the female gut.

The findings suggest unusual metabolic roles for proline and give further rationale for the trial of antiproline drugs for schistosomiasis.

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Sperm activation and fertilization in Limulus polyphemus. Ross L. Shoger and David W. Bishop.

Electrically spawned spermatozoa are in dense suspension and nonmotile. Dilution to 0.05% in artificial or natural sea water (SW) fails to elicit motility. However, addition of milky oviducal exudate, obtained coincident with eggs by electrical stimulation, induces vigorous sperm motility which lasts 4–5 minutes. Similarly, SW in which eggs have resided, pH 4.2 SW-egg extract readjusted to pH 8.0, and acid-alcohol egg extract elicit sperm motility when mixed in equal proportions with 0.5% sperm suspension in MBL SW. Egg water activating factor is heat-stable and dialyzable. Exposure of sperm to pH 4.9 to 9.0 SW or *Arbacia* egg water fails to stimulate motility. A 0.02% SW solution of Haemo-sol, a laboratory detergent, stimulates intense motility, but deoxycholate is ineffective. While EDTA-SW elicits sperm motility, studies with combinations of 0.01 *M* EDTA solutions and sperm suspensions, made up in Ca-free, Mg-free or Ca-Mg-free SW, suggest that Mg is important in the motility response. Quiescent sperm suspensions, previously activated with egg water or EDTA, may be restimulated for a second, briefer period with additional activating solution.

Aging in SW produces a high incidence of false acrosomal reactions, described by André (1963), but almost no true acrosomal reactions. Egg water produces less than 0.5%, acid-SW egg extracts approximately 5%, and acid-alcohol extract about 10% of true acrosomal reactions. Addition of 10–20% 0.5 *M* $CaCl_2$ to 0.5% SW sperm suspension produces approximately 6% true acrosomal reactions without stimulating motility. Almost 100% false acrosomal reactions result from suspension of sperm in double strength SW.

When fresh, electrically spawned eggs are placed in 2.5, 0.5, or 0.05% sperm suspension, up to 97% activation results. Developing embryos appear viable histologically although none has as yet hatched from the chorion after 4 weeks under laboratory conditions.

Electron microscopic studies of the inseminated egg are in progress.

This study was made in the Fertilization and Gamete Physiology Training Program (NIH grant T1 HD-26-06).

Structure and function of the mesoglea in a stauromedusan. Elnora H. Smith and Hugh Y. Elder.

It has long been known that coelenterate mesoglea contains collagen fibers. Recent literature suggests that the mesoglea of stauromedusae differs from that of polypoid coelenterates by the possession of elastic fibers. *Craterolophus convolvulus* were fixed, sectioned, and stained (notably with spirit blue/Van Giesen) for light microscopy.

Collagenous material comprises a network which forms a narrow dense layer under the epidermis, a broader dense layer under the gastrodermis, and a more diffuse meshwork between. The arrangement is strikingly similar in both longitudinal and transverse sections. The collagen fibers therefore appear to form a three-dimensional network capable of distension radially, longitudinally, or circumferentially. This geometrical configuration has definite limits of distension, and since it is formed of inextensible fibers, the system imposes limits on the animal's range of shape.

Stout elastic fibers, thickest in the middle of the mesoglea, and fanning out in arborescent manner under the epidermis and gastrodermis, are found running approximately radially through the mesoglea. These fibers and their branches are often sinuous—largely an artifact of processing shrinkage (the radial fibers in hand-cut sections of fresh tissue are almost straight). This kinking is not fortuitous but due to close binding of radial elastic fibers to the collagen system.

The stalk and bell of the stauromedusae have only longitudinal muscles. If the animal maintains constant volume during contraction of these muscles, the body wall must expand both radially and circumferentially. Clearly the elastic fibers would oppose radial distention; additionally, through linkage with the collagen network, they also oppose circumferential distension. Therefore the arrangement of radial elastic fibers allows efficient antagonism to longitudinal muscles.

This system strikingly contrasts that of the Anthozoa, where a stout three-dimensional collagen meshwork also occurs, but radial elastic fibers are absent and circular muscles are present.

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Nicotine effects on the gametes of Arbacia punctulata. CARL CASKEY SPEIDEL AND RALPH HOLT CHENEY.

The chief purpose of this study was to observe and record in motion pictures the structural changes in developing sea urchins following nicotine treatment of eggs alone or sperm alone. It has long been known that nicotine in suitable dosages can profoundly affect the surface of the sea urchin egg in such a way as to allow polyspermy. Just how nicotine may affect the spermatozoon has not been analyzed adequately.

Gametes of each type were exposed to nicotine solutions of nine different strengths ranging from 0.5 to 0.00002 molar. Exposure times ranged from 1 to 24 hours. Nicotinized gametes were fertilized with normal gametes of the opposite type. Significant abnormalities soon became visible in the progeny. Six hours after fertilization, retarded and defective cleavage varieties were conspicuous. Two days after fertilization, when motile plutei were the dominant form in normal control cultures, the progeny, whether from suitably nicotinized eggs alone or nicotinized sperm alone, consisted largely of retarded and abnormal individuals. Pronounced skeletal deficiencies were visible in many. Effects included animals with arms of different lengths or with no arms. Such skeletal defects were accompanied by abnormal motility. Defective non-skeletal structures were also noted. Our experiments also revealed that very strong dosages to sperm alone, as well as to egg alone, could prevent development after fertilization, death occurring in most fertilized cells before the first or second cleavage.

This indicates that nicotine treatment of the egg damages both cytoplasm and nucleus, whereas nicotine treatment of the spermatozoon damages only the nucleus. A much stronger dose to sperm than to eggs, therefore, is necessary to induce approximately equal damage to the progeny. These results form an interesting comparison with those reported previously by us on the effects of caffeine, colchicine, trichloroacetic acid and radiation.

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Chromatographic separation of a cardioexcitatory substance extracted from M. mercenaria. MORRIS A. SPIRTES AND DAVID JACOBOWITZ.

Evidence for a cardiostimulatory compound other than serotonin in molluscan organ extracts has recently accumulated. Since the clam heart is used as a test organ for cardioactive compounds it was of interest to examine it for the presence of such a compound. Extracts of hearts were made after homogenization in 0.1 N HCl, centrifugation (1 hr., 100,000 *g*) and lyophilization. The lyophilized material or the acid extract was streaked on thin-layer, silica-gel chromatograms, run in butanol/acetic acid/H_2O (4:1:2) for 10 cm. and dried overnight. Attention was focused on a ninhydrin-positive, UV-fluorescent region (R_f 0.27–0.37) that gave cardioexcitatory effects in 30% of the extracts examined and cardioinhibitory effects in 60%, when tested on an isolated *M. mercenaria* heart. This region thus appeared to contain a mixture of at least two compounds, one cardioinhibitory and the other cardioexcitatory and not located in the R_f 0.71 region of serotonin. Further separation was attempted by column chromatography using gel filtration (Sephadex G-25). Two cardioactive fractions could be eluted with sea water. The first, a cardioinhibitory fraction, became excitatory following treatment with acetylcholinesterase at 37° for 30 min. The same inhibition could be blocked by pretreatment with benzoquinonium (10 μg./ml.) and again a cardioexcitatory action sometimes lasting for 2 hours was revealed. The second fraction, closely following the first, caused long-lasting cardioexcitation. This was not blocked by pretreatment of the isolated heart by methysergide (10 μg./ml). Thus, both separation methods resulted in the separation of (a) a cardioinhibitory fraction, probably acetylcholine, and (b) a cardioexcitatory fraction which does not appear to be serotonin, because of its behavior on thin-layer chromatography and the fact that its activity cannot be blocked by lysergic acid derivatives. The nature of the latter is as yet unknown.

"Swimmerette," a new sex-linked recessive mutant in the brine shrimp, Artemia salina Leach. R. D. SQUIRE AND D. S. GROSCH.

"Swimmerette" (*sm*), a new mutant, was discovered in an inbred line of *Artemia* derived from Utah cysts. The gene is an X-linked recessive with incomplete penetrance (30–50%), and variable expressivity in the homogametic male (XX). In the heterogametic sex, XY females, the trait is rarely observed. The swimmerette phenotype, which in simplest form is reminiscent of a small unspecialized crustacean appendage, involves an apparent fusion, or alternatively a failure of separation, of adjacent "appendages." This fusion may include any adjacent pair or trio of phyllopodia, or involve phyllopodia and genitalia. Occasional cyclopean individuals with fused or almost-fused eyes also occur. Expression ranges from just barely detectable fusion at the bases of the appendages to fusion of the entire structures. These abnormalities are usually asymmetrical but may occur more than once in a given animal. Data have been obtained from crosses with Bowen's partially sex-linked mutant "white eye." A double mutant line "sm w" has been established which combines "swimmerette" and Bowen's locus in one strain.

Supported by NASA grant NsG-678.

Intertidal activity of Uca pugilator. FOWLER M. STILLMAN AND JONATHAN P. GREEN.

The intertidal area studied, a section of sandy beach on the Herring River, West Harwich, Mass., experiences semidiurnal tides. Individual crabs marked with colored tape were observed.

Feeding variations exist between sexes, females processing 50% more food-bearing sand per unit time than males. Males require longer feeding periods than females to obtain an equivalent amount of food, due to their having only one small cheliped while females have two.

Counts of crabs showed sex ratios varying with beach zone and exposure time. Males and females feed in equal numbers for about five hours on the lower beach; then females migrate to the burrow area. Males do not migrate until the incoming tide forces them up the beach where they may oust females and smaller males occupying open burrows. Ousted crabs seek another burrow. Migrating and ousted males sometimes line up to fight the resident of a burrow, but male residents so challenged retain the burrow 80% of the time. Ousted females, if other burrows are occupied, dig new ones near the rising tide.

Migrations of a small population of crabs were followed daily. Although crabs do not return to the same burrow every day, they do migrate to the same general feeding and burrow areas. The crab's "territory" is the burrow fought for at each rising time. It doesn't defend a specific burrow for any length of time, so it doesn't show classical territoriality.

Height of tides, time of low tides, and weather conditions influence burrow location by affecting sand moisture content. Migration of crabs is related to the length of the feeding period. It is hypothesized that burrows are primarily located within a zone the upper limit of which is controlled by sand moisture content and the lower limit by feeding time, determined by the incoming tide.

Scintillomicroscopic observations of L-proline-U-C^{14} uptake in Schistosoma mansoni. B. L. STRUNK, G. T. REYNOLDS AND A. W. SENFT.

Principles previously discussed (Reynolds, Strunk and Senft, 1967) have been tested and confirmed in observations on the parasite *Schistosoma mansoni.* Schistosomes incubated in nutrient medium containing L-proline-U-C^{14}, in concentrations ranging from 2 to 10 microcuries per milliliter, over periods ranging from 2 to 10 hours, were mounted on a slide, and covered with a thin scintillating material that gave out light in response to the electrons from the C^{14} decay.

The desirable properties for the scintillator include: small electron scattering cross-section, transparency, short decay time for light emission, and a high efficiency for converting the energy of the radioactive decay product into photons. Observations have been made using a plastic scintillator with good transparency but an efficiency of only a few per cent; silver-activated ZnS, a high efficiency, non-transparent scintillator; and sodium-activated CsI which was transparent, but had prohibitively low scintillation efficiency in the thin crystals used.

In the present work schistosomes were focussed on the first cathode of the image intensifier system through optics, providing magnifications of 1 ×, 4 ×, 10 ×, and 22 ×. Typical photon collection percentages ranged from 0.3% to 3.0%. In one test, an intensifier with a fiber optics input window was used. This method is restricted to 1 × magnification, but was impressive in that useful pictures of radioactivity resulted from exposures as short as a few hundredths of a second. At higher magnifications exposures up to several minutes were used.

Several important results were obtained by the method. Proline was found to concentrate in two regions in the anterior of the male. One region was in the proximity of the testes and the other region was near the neural ganglia. Proline was not found to concentrate nearly as heavily in the female, and was found to be noticeably absent from the posterior of the female, which contains the vitelline glands. No radioactivity has been localized in females that were not in copula with a male during incubation.

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The life-cycle and developmental stages of a digenetic trematode whose unencysted metacercarial stages occur in medusae. HORACE W. STUNKARD.

Distoma bacillare Molin, 1859, was designated type of a new genus, *Opechona,* by Looss, 1907. A dozen or more species of *Opechona* have been described from various fishes in different parts of the world, but the accounts are so divergent that the diagnosis of the genus and validity of certain described species are dubious. Nicoll (1910) redescribed *Pharyngora bacillaris* (= *Opechona bacillaris*); stated that the species was common in *Scomber scombrus* and *Gadus merlangus* from British waters; and reported a larva, found in a plankton net, agreed so completely with juvenile specimens from fishes that he was "practically certain" of their identity. Marie Lebour (1916) reported unencysted larvae of *P. bacillaris* in medusae of *Obelia* sp., *Cosmetira pilosella, Turris pileata,* and *Phialidium hemisphericlium,* and in the ctenophore, *Pleurobrachia pileus.* Lebour (1917) recorded these metacercariae from *Sagitta bipunctata,* together with the description and figure of a cercaria identified as the larva of *P. bacillaris.* Unencysted larval trematodes occur in hydrozoan and scyphozoan medusae of the Woods Hole area. Dr. Frank Fisher found them in the medusae of *Bougainvillia carolinensis* and we have taken them in *Gonionemus vertens* and *Chrysaora quinquecirrha.* Cercariae with long, setigerous tails occur in *Anachis* sp.; they penetrated the medusae of *G. vertens* and *C. quinquecirrha* in enormous numbers. Through the good offices of Mr. Charles L. Wheeler, Director of the Aquarium, various fishes were exposed to experimentally infected *G. vertens* and *C. quinquecirrha.* Three of three exposed *Stenotomus chrysops* yielded 92 worms, ranging from juveniles to gravid adults. These worms belong to *Opechona* or a closely related genus, but final allocation depends on further information. The number and pattern of flame-cells and penetration glands in the cercaria are yet uncertain.

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Shark antidiuretic factor: Inhibition of water diuresis in the dogfish, Mustelus canis, by extracts of homologous brain and spinal cord. RICK I. SUBERMAN AND LAWRENCE RABINOWITZ.

Elasmobranchs are osmotically more concentrated than their sea water environment, taking up solute-free water through their skin and gills and losing it by virtue of the renal excretion of a urine which is always hypo-osmotic to both plasma and sea water. When placed in dilute sea water or when migrating to fresh water, increased water uptake leads to a reduction of plasma osmolality, a reduction in the urine/plasma osmolality ratio (U/Posm), a rise in glomerular filtration rate (GFR), and the excretion of larger volumes of urine. Speculations that this response to dilution is mediated by endocrine or neuroendocrine mechanisms led us to test the effect of extracts of homologous spinal cord and brain on the renal function of *Mustelus canis* undergoing a water diuresis while maintained in 75% sea water.

Donor fish were maintained in 75% sea water for 2–3 days and then tissue (brain, spinal cord, muscle or liver) was removed, homogenized, mixed with dogfish Ringer's, and centrifuged at 500 or 12,000 *g*. When 4 ml. of Ringer's (1 fish) or of supernatant of liver (2 fish) or muscle (1 fish) were injected intravenously, no consistent or significant changes in urine flow, GFR (inulin clearance), or U/Posm occurred when compared with pre-injection control values from the same fish. In the two-hour period after injection of 4 ml. of supernatant of brain (2 fish) or spinal cord (3 fish) large and significant decreases in urine flow (59–79%) and GFR (55–80%) were always observed. U/Posm always increased significantly (5–37%). Blanching of the tail and darkening of the blood also were observed after injection. Urine flow, GFR and U/Posm returned towards pre-injection levels in the succeeding 4–10 hours after injection in some fish, but remained altered in others during this period.

Reduction of GFR and evidence suggestive of peripheral and gill vasoconstriction indicate the presence of vasoconstrictor substances in brain and cord extracts. Presence of factors affecting the permeability of the nephron to water is also suggested by the observed increases in U/Posm. Although these extracts contained antidiuretic factors which reversed the changes seen during dilution, we can not state with certainty that these factors play a role in the normal physiological control of renal function. Since vasopressin, oxytocin and dogfish pituitary extracts do not inhibit water diuresis in *Squalus acanthias* (Truniger, Schmidt-Nielsen and Rabinowitz, unpublished observations) and epinephrine causes a diuresis in the dogfish, these compounds are excluded as candidates for the antidiuretic factor. Many others remain.

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Winter distribution of Loligo pealei determined by exploratory trawling. WILLIAM C. SUMMERS.

Three cruises were made to the area between Hudson Canyon and Cape Hatteras in December, February and April of the winter of 1966–1967. Approximately 150 stations were made towing #41 otter trawls at 3.5 knots (6.5 km./hr.); 4 metric tons of *Loligo pealei* were taken at 86 of these stations and a total of 4000 individual squid were sexed and measured. The stations were situated on transect lines across the continental shelf and extended to 1500 m. depth. Preliminary investigation has shown:

(1) Approximately 97% of the squid caught were *L. pealei,* the remainder were mostly *Illex illecebrosus.*

(2) The sex ratio was close to 1:1 in every sample and the larger size of mature males was noted.

(3) Individual net hauls produced squid of consistent size. The majority of the hauls included the central two-thirds of the sample number within the interval 0.8 to 1.2 times the median mantle length.

(4) The median size was directly proportional to the depth from which the sample was taken. This relationship varied with the mesh size of the cod end liner.

(5) The squid catch was approximately normally distributed with sampling depth. A fit normal curve has a mean of 128 kg. of *L. pealei* per hr. at 170 m. depth and a standard deviation of 75 m.

(6) The distribution of *L. pealei* was not obviously correlated with latitude or bottom temperature.

(7) It is estimated by extrapolation that the winter population of *L. pealei* between Hudson Canyon and Cape Hatteras has a magnitude of 1 billion individuals.

I wish to acknowledge the cooperation of the Bureau of Commercial Fisheries and the Fisheries Research Board of Canada for the use of cruise data from the R/V Delaware (#66-11 and 67-1) and the A. T. Cameron (#130) respectively.

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Chromosomal basic proteins in sea urchin eggs, sperm and embryos. M. MICHAEL THALER, RALPH P. FRANCESCONI AND CLAUDE A. VILLEE.

The gross composition of histones, the basic proteins associated with chromosomal DNA, is remarkably similar in the most widely separated phyla. However, changes have been demonstrated in these proteins during embryonic development, and during periods of active DNA synthesis. Histones were therefore extracted from unfertilized eggs, late gastrula embryos, and sperm of *Arbacia punctulata,* and analyzed for electrophoretic mobility and amino acid content. Highly purified suspensions of nuclei (contamination 1%) were used to prepare chromatin from which the acid-soluble proteins were extracted. Disc electrophoresis revealed similarities in mobility and band distribution when compared with similarly extracted proteins from sea urchin sperm and calf thymus. In all preparations six bands were present in a constant arrangement of three minor, two major, and one minor bands. Basic residues predominated over acidic residues in late gastrula embryos and sperm by ratios of 1.33 and 1.43, respectively, while the ratio in unfertilized eggs was 0.93. A trace of E-N-methyllysine (—0.01 mole%) was found in histones from embryos and only traces of cysteine, methionine, and tyrosine were present in all three preparations. The most striking developmental effect was in the lysine/arginine ratio which was reduced from 8.38 in unfertilized eggs to 0.57 in late gastrula embryos, reflecting a rise in the absolute value of arginine from 1.63 moles% to 15.11 moles%, and a decrease in lysine from 13.66 moles% to 8.54 moles% during the first twenty hours after fertilization. In summary, basic proteins extracted from chromatin of sea urchin eggs, embryos, and sperm fit the criteria for mammalian and plant histones. Considerable changes in lysine and arginine content occur in the histones of fertilized sea urchin eggs. The concentrations of other amino acids are relatively stable during early development and are present in proportions equivalent to those found in histones from other phyla, such as calf thymus and pea-bud fraction 2b.

The intracellularly recorded response in the scallop eye. JUN-ICHI TOYODA AND ROBERT M. SHAPLEY.

Intracellular recording was attempted with a microelectrode after the removal of the cornea and lens of the excised scallop eye. One type of response was obtained relatively stable for several minutes. There is a resting potential of —30 to 40 mV., a hyperpolarization during illumination, and a slight depolarization at off of illumination sometimes with small spikes superimposed on it. The response to bright light shows an initial transient of up to 40 mV. which decays with time to relatively steady-state. As the light intensity is lowered, the transient becomes less marked and is absent below about 1 log unit intensity above the threshold. Resistance measurement with a bridge circuit shows that both the hyperpolarization during illumination and the depolarization at off of illumination are associated with a conductance increase of the membrane. A similar off rebound was also produced by hyperpolarizing current instead of light. The estimated equilibrium potential for the hyperpolarization was —70 to 80 mV. The immersion of the preparation in tetrodotoxin (5×10^{-8} g./ml.) or in Na-free solution for 30 minutes did not affect the size of the response recorded, suggesting that this hyperpolarization is not a synaptic potential produced by action potentials in the proximal cells.

The work was supported, in part, by a research grant (B864) from the National Institute of Neurological Diseases and Blindness, U.S.P.H.S.

Oxygen binding and quaternary structure of hemocyanin. K. E. VAN HOLDE AND HENRY A. DEPHILLIPS, JR.

For some time it has been suspected that multi-subunit proteins which are capable of cooperative binding of oxygen might exhibit a dependence of quaternary structure upon oxygenation. We have obtained clear evidence for this phenomenon in the hemocyanin of the squid *Loligo pealei.* Ultracentrifuge studies have been performed upon deoxygenated, partially, and wholly oxygenated hemocyanin solutions, containing 0.01 M Mg^{++}, over the pH range from

6.3 to 8.02. The results may be summarized as follows: (1) deoxygenated hemocyanin is in the 59S ($M = 3,800,000$) form over the entire range. (2) *Partially* oxygenated solutions are partially dissociated to the 19S ($M = 770,000$) and 11S ($M = 375,000$) subunits. This is observed only over a narrow pH range, because of the sharpness of the Bohr effect. (3) Wholly oxygenated samples are in the 59S form. The data are most easily explained by the hypothesis that *partially* oxygenated subunits are incapable of association.

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Bi-ionic action potentials in squid giant axons intracellularly perfused with sodium salts. AKIRA WATANABE, ICHIJI TASAKI AND LAWRENCE LERMAN.

Squid axons, intracellularly perfused with suitable salts of univalent cations, maintained excitability in external media containing the salt of a divalent cation as the sole ionic species. Action potentials evoked under these extremely simple ionic conditions may be appropriately termed "bi-ionic action potentials." Suitable internal perfusates included phosphate salts of Li^+, Na^+, Cs^+, TMA^+, TEA^+, TPA^+, choline, ethylamine, ethanolamine, guanidine, and methylguanidine. In most cases, production of all-or-none action potentials of long duration (0.1 to 20 sec.) could be maintained for over 1 hour in external media containing 0.1 *M* $CaCl_2$. The bi-ionic action potential obtainable with 0.1 *M* $CaCl_2$ externally and 0.03 *M* Na-phosphate internally was 40–60 mV. in amplitude and 0.1–15 sec. in duration. The resting potential was about −20 mV. under such conditions. Upon addition of 0.03 *M* NaCl to the external medium, the intracellular negativity fell slightly, the action potential amplitude increased by several mV., and an overshoot of approximately 20 mV. was observed. Large bi-ionic action potentials (80–120 mV. in amplitude) could be produced with 0.025 *M* choline phosphate or TEA-phosphate internally. The typical long duration action action potential produced under bi-ionic conditions could be converted to a "normal" action potential (1 msec. duration and 100 mV. amplitude) by switching the internal medium to 0.4 *M* KF and the external medium to 0.3 *M* NaCl and 0.1 *M* $CaCl_2$. This reversibility indicates that the bi-ionic conditions produced no permanent alteration of the axon membrane. The experimental findings can not be explained by a theory of excitation which maintains that a specific increase in Na-permeability and influx of Na^+ into the cell is the primary cause of excitation or that the peak of the action potential is determined by the Na^+ concentration ratio across the membrane. The findings support the view that a cooperative ion exchange process between univalent and divalent cations at negatively charged sites of the membrane is the primary event in excitation.

The presence of a dynein like ATPase in the soluble phase and in the isolated mitotic apparatus of sea urchin eggs. RICHARD WEISENBERG AND E. W. TAYLOR.

Recent work by Inoué and others has indicated that the mitotic apparatus is formed from proteins present in the soluble phase of the cytoplasm. If the mitotic apparatus contains an ATPase, then one would expect to find during at least some portion of the cell cycle an ATPase in the soluble phase of a mitotic cell. It has been found that there is an ATPase present in a four-hour 100,000 *g supernatant* of fertilized or unfertilized sea urchin eggs homogenized in an isotonic sucrose-Mg^{++} medium (1 *M* sucrose, 5 m*M* $MgCl_2$, 0.05 *M* Tris, pH 7.5). The specific supernatant activity is highest with the fewest passes through the homogenizer and is uneffected by large changes in ionic strength, $MgCl_2$ concentration, and osmolarity of the homogenizing medium. The soluble-phase ATPase has been analyzed by zone centrifugation on sucrose gradients and found to migrate as a single major peak with a sedimentation coefficient of about 14S. The ATPase is activated almost equally by Mg^{++} and Ca^{++}, hydrolyzes ATP much better than GTP or ITP, and has pH optima at about pH 7 and pH 9. These properties are similar to those of dynein, the ATPase isolated from cilia and sperm tails by Gibbons.

ATPase activity has also been found in the mitotic apparatus isolated from *Strongylocentrotus* eggs by the method of Kane. Part of the activity is readily soluble in either 0.05 *M* KCl or in 0.05 *M* Tris, pH 7.5. The soluble activity migrates on a sucrose gradient in a single symmetrical peak with a sedimentation coefficient of about 14S. The specific ATPase activity extractable from the mitotic apparatus, however, is not significantly higher than that which can be extracted by appropriate treatment of the egg residue remaining after removal of the mitotic apparatus. It is possible, therefore, that the mitotic apparatus ATPase is not a specific mitotic apparatus protein but a general cytoplasmic constituent of the egg.

This study was made in the Fertilization and Gamete Physiology Training Program (NIH grant T1 HD-26-06).

A study of dogfish corneal collagen. S. ZIGMAN, S. LERMAN AND S. BETLEM.

Approximately 60% of the dry weight of the cornea is collagen. A characteristic of corneal aging is the conversion of soluble to insoluble collagen by an increase in intermolecular cross-linking. It was found that with aging the amount of NaCl-soluble collagen in the dogfish (*Mustelus canis*) cornea diminishes markedly, while the amount of insoluble collagen increases.

The influence of aging on the *in vitro* synthesis of the collagen fractions of these corneas was studied by incubating (at 20° C. in elasmobranch Ringer's solution containing C^{14}-proline under 95% O_2:5% CO_2) groups of 20 corneas of freshly killed fish. The corneas were frozen, homogenized in distilled water (Virtis homogenizer) and fractionated by a 48-hour dialysis extraction with 0.5 *M* NaCl followed by a similar extraction with 0.5 *M* HAc. The insoluble collagen residue was solubilized by gelatinization for 3 hours in an autoclave. The collagens were precipitated (10% NaCl + 0.5 *M* HAc), redissolved in 0.5 *M* HAc and lyophilized. Portions of the lyophilized fractions were hydrolyzed, and the specific radioactivity of C^{14}-hydroxyproline was used as a measure of collagen synthesis. Actinomycin and puromycin effects were studied.

Collagen synthesis (20-hr. incubation) was greatest in the NaCl-soluble collagen of young fish corneas (20–30 mg. each.) Little incorporation into HAc-soluble or into insoluble collagen was found in either young or old fish corneas (120 mg. each). When 40 μg./ml. of actinomycin D was added to the incubation medium, synthesis of NaCl-soluble collagen was depressed in both young and old fish corneas by 30–40%, but no effect was observed on the insoluble collagen. It appears that *in vitro* collagen synthesis occurs mainly in the NaCl-soluble fraction, and at a higher rate in young than in old fish corneas. An apparent dependence on a stable messenger-RNA is suggested.

After further purification of NaCl-soluble collagen by dialysis against 0.05 *M* Na_2HPO_4, electron microscopy showed a ribbon-shaped, cross-striated, fibrous material (diameter 5 μ). Ultracentrifugation at 50,740 rpm indicated a sedimentation rate of 2.6S. Amino acid analysis indicated proline at 11.7%, hydroxyproline at 6.9%, and glycine at 30% (similar to dogfish skin collagen). Denaturation at 40° C. gave an acrylamide gel electrophoresis banding pattern with a large β component, a small γ component, and an intermediate-sized α component.

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Preliminary analysis of Thalassopsammon along a transect from high-tide line to a point 650 feet directly southwest off Nobska Beach, Woods Hole, Massachusetts. DONALD J. ZINN.

Paired cores in 25-cm. numbered, clear plastic tubes, 35 cm. in diameter, were collected at low tide on August 1 at 50-foot intervals along a transect from a point 650 feet (16.5 feet deep) southwest of Nobska Beach, Woods Hole, Massachusetts, to the high-tide line of the beach. The offshore collections were made by two SCUBA divers who handed them to assistants on board a shallow-draft research vessel. Intertidal cores were taken both horizontally and vertically 12 feet apart.

In the laboratory, cores were refrigerated at 10° C. until examined. The air-bubbling technic was used to remove protozoa and micrometazoa from the sediment; all individuals, after separation into major groups, were counted, recorded and preserved. Sediments from every sampling station were sieved and analyzed using standard technics for granulometry.

Preliminary results of this investigation may be summarized as follows:

(1) No correlation appears between the Phi values of sediment particles and either the numbers or the variety of the higher taxa of the interstitial populations.

(2) With the possible exception of the Turbellaria, variation in the amount of interstitial detritus did not seem to affect the occurrence of the higher taxa.
(3) Mystacocarida and Gastrotricha were found only intertidally; Cumacea, Amphipoda and Archiannelida were collected solely below low-tide line; Acarina, Copepoda, Turbellaria, Nematoda and Polychaeta decreased in total numbers from low- to high-tide lines.
(4) An increase in numbers of individuals as the beach is approached from offshore was recorded from Nematoda, Copepoda, Acarina and Turbellaria; the reverse trend was observed in Polychaeta, Foraminifera, and Archiannelida.
(5) Clumping was noticed particularly among Oligochaeta, Ostracoda and Turbellaria.
(6) Large numbers of micrometazoa per station were found intertidally (764/100 gm.) then subtidally (218/100 gm.); the largest accumulation of organisms was just above the low-tide line.
(7) A previously recorded tendency in Nobska and other beaches appeared in *Thalassopsammon* populations along this transect when it was observed that copepods and nematodes were present in opposite concentrations at nearly every station.

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THE UPTAKE OF NA-22 DURING INDUCTION IN PRESUMPTIVE EPIDERMIS CELLS OF THE RANA PIPIENS GASTRULA[1]

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Previous investigations (Barth, 1966) have shown that induction of various cell types from presumptive epidermis cells of the *Rana pipiens* gastrula by sucrose is a two-step process. The sucrose solution prepares the cells for induction by the salt solution in which they are cultured. Induction by the salt solution after sucrose treatment is proportional to the concentration of sodium chloride. The induction produced by lithium chloride in high concentrations for relatively short periods of time is likewise dependent upon the concentration of sodium chloride in the solution used for culturing the cells (Barth and Barth, unpublished data). While our standard solution contains other ions which may possibly complete the process of induction started by sucrose or lithium chloride, the sodium chloride dependency of the process suggested a study of the uptake of Na^{22}. Are the cells so altered by treatment with sucrose or lithium chloride that, upon return to standard solution, sodium ions enter more rapidly than in control cells which have not undergone treatment?

METHODS

The procedure for isolating, treating and culturing small aggregates of cells from the gastrula has been described in earlier publications (Barth and Barth, 1967, for references). The concentration of NaCl in the standard solution used for operating is 515 mg. per 100 ml. For treatments of cells the medium was modified as described in detail in tables of data presented below. When aggregates were cultured in order to observe effects of treatments upon differentiation, the NaCl content of standard solution was 450 mg. per 100 ml.

An important aspect of the method for preparing presumptive epidermis cell aggregates is the use of Versene (EDTA) to loosen the superficial pigmented surface coat layer from the underlying presumptive epidermis cells. Only the latter were used in experiments, since the pigment coat layer of the anuran egg is relatively impermeable to most compounds.

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Most of the experiments reported below involved treatments of presumptive epidermis cells known to bring about induction of new cell types, followed by measurement of the influx of Na^{22}. The isotope was presented either together with the inductor (sucrose or the lithium ion), or following pre-treatment with the inductor. When the lithium ion was used as inductor, a positive correlation was observed between concentration of LiCl used for treatment and whether or not presumptive epidermis cells were induced to differentiate into a new cell type. Preliminary experiments indicated that both 2- and 4-hour treatment with LiCl at a concentration of 600 mg. per 100 ml. of standard solution (modified to contain no NaCl) induced extensive pigment ring cells, which are the precursors to melanocytes. When the concentration of LiCl was reduced to 300 mg., presumptive epidermis cells merely formed ciliated spheres of epidermal cells. Uptake of Na^{22} following 4 hrs. treatment in 300 mg. LiCl was only 214 counts per minute (c.p.m.) as compared with 355 at a concentration of 600 mg. LiCl per 100 ml. At 2 hrs. treatment the effect of increasing LiCl treatment on Na^{22} uptake gave values of 144 c.p.m. with 300 mg. LiCl and 260 c.p.m. following treatment with 600 mg. LiCl. These preliminary experiments indicated that induction of new types of differentiation, as well as uptake of Na^{22}, both were proportional to concentration of the lithium ion used as inductor. In experiments presented below, LiCl was used at concentrations known to induce new cell types.

Following exposure to the isotopes, aggregates were washed through three changes of unlabelled standard solution and pipetted, together with a minimal volume of solution, into aluminum planchets. Samples from the third wash solution were pipetted into other planchets to check for carry-over of isotope not bound to the cells. Values measured for the third wash were similar to the background c.p.m.

Planchets bearing the samples were dried in an oven at 110° C. for 30 min. and counted on a Model 186 Nuclear-Chicago low background counter. Counts per minute were calculated by averaging the 5-minute readings. Finally c.p.m. were expressed in terms of 200 aggregates, calculated from actual values read for samples consisting of 95 to 215 aggregates. Each aggregate consists of approximately 125 cells.

The isotopes used, obtained from New England Nuclear Corporation, were sodium^{22}, HCl solution, carrier-free (neutralized before use with $KHCO_3$), and uridine-2-C^{14} (specific activity 20–30 mC./mM). Sodium^{22} was diluted to a final concentration of 1.5 μC. per ml. in standard solution. Uridine-C^{14} was used at a concentration of 0.1 μC. per ml. of standard solution.

RESULTS

1. *Uptake of Na^{22} when sucrose is used as an inductor*

Preliminary experiments showed that Na^{22} added to sucrose was taken up by the aggregates of presumptive epidermis. After the Na^{22} had reached an equilibrium value, the aggregates were returned to standard solution containing Na^{22} and the isotope left the aggregates.

When Ca^{++} was added to the sucrose the uptake of Na^{22} was reduced, as shown in Table I. The concentration of Ca^{++} was approximately that of our standard salt solution. Since Ca^{++} generally brings about a decrease in permeability of the cell

TABLE I

Effect of sucrose and of calcium on uptake of Na-22 by aggregates of presumptive epidermis cells

Concentrations of compounds were 58 mg./ml. sucrose; 0.2 mg./ml. $CaCl_2 \cdot 2\ H_2O$; and 1.5 μC. Na^{22}/ml.

Experiment No.	Compounds	Hours	No. of aggregates	c.p.m.	c.p.m. for 200 aggregates
1	Sucrose	1.5	177	3495	3950
	Sucrose + Ca	1.5	177	541	612
2	Sucrose + Ca	1.75	185	290	314
	Sucrose + Ca	3.75	185	304	329
3	Sucrose	0.5	185	650	703
	Sucrose	1.5	185	2148	2330

surface, the decreased uptake of Na^{22} appeared to be the result of a decrease in the permeability which was present in sucrose. The data in Table I could be best interpreted to demonstrate an increase in permeability of the aggregates in sucrose.

The next series of experiments was designed to determine whether the increased permeability caused by treatment with sucrose would lead to an increased uptake of Na^{22} after the aggregates were returned to standard solution. A comparison of Na^{22} uptake by aggregates which were treated with sucrose and by aggregates which were treated with standard solution is given in Table II. After treatment with sucrose and standard solution the aggregates are transferred to a solution containing Na^{22} and the uptake determined after varying periods of time. Sucrose-treated aggregates take up more Na^{22} than do control aggregates treated with standard salt solution containing 4.50 or 5.15 mg. NaCl per ml. Thus the sucrose treatment alters the cell surface so that Na^+ enters more rapidly. The largest differences between sucrose-treated aggregates and controls are found for the shorter periods of exposure to the isotope. Therefore the change in the cell surface produced by sucrose treatment may undergo recovery when the aggregates are transferred to standard salt solution. On the other hand, the sucrose-treated aggregates in Na^{22} may simply reach an equilibrium more rapidly. More information on this question will be found in the section dealing with Na^{22} uptake with Li^+ as the inductor.

2. *Uptake of Na^{22} when LiCl is used as an inductor*

Preliminary experiments showed an increase in uptake of Na^{22} with an increase in the concentration of LiCl. This suggested an alteration of the cell surface by LiCl. Since the induction by Li^+ is dependent upon the concentration of NaCl in the solution to which the Li^+-treated aggregates are returned, experiments were designed to measure the uptake of Na^{22} after Li^+ treatment.

Table III records the uptake of Na^{22} by aggregates which have been treated with lithium as compared with the uptake of controls. The results are similar to those recorded in Table II where sucrose is used as the inductor. More Na^{22} is taken up by those aggregates which have been exposed to lithium for 2.0 hours as compared with those which have been kept in standard solution for 2.0 hours.

TABLE II

Effect of sucrose pretreatment on uptake of Na-22 by presumptive epidermis cells

Concentrations of Na refer to mg. NaCl per 100 ml. standard solution.
Concentration of sucrose is 5.8 grams per 100 ml. glass-distilled water.

Exp. No.	Treatment		Post-treatment			No. of aggs.	c.p.m.	c.p.m. 200 aggs.	Difference ± sucrose
	Concentration	Hrs.	Conc.	Hrs.	μC. per ml.				
1	450 Na	1.5	450 Na	3.5	1.5	180	45	50	32
	Sucrose	1.5	450 Na	3.5	1.5	180	74	82	
2	450 Na	1.5	450 Na	1.25	1.5	155	35	45	44
	Sucrose	1.5	450 Na	1.25	1.5	155	69	89	
3	515 Na	1.5	257 Na	2.0	1.5	215	31	29	24
	Sucrose	1.5	257 Na	2.0	1.5	215	57	53	
4	515 Na	1.5	257 Na	0.5	1.5	145	37	51	54
	Sucrose	1.5	257 Na	0.5	1.5	145	76	105	
	515 Na	1.5	257 Na	1.5	1.5	116	44	76	5
	Sucrose	1.5	257 Na	1.5	1.5	116	47	81	
5	515 Na	1.5	450 Na	0.5	1.5	100	15	30	40
	Sucrose	1.5	450 Na	0.5	1.5	100	35	70	
	515 Na	1.5	450 Na	1.0	1.5	115	30	52	23
	Sucrose	1.5	450 Na	1.0	1.5	115	43	75	

In the next series of experiments (Table IV) the uptake of Na^{22} was measured during the period of lithium treatment. The NaCl content of the standard solution was lowered to 2.57 mg./ml., and 3.0 mg. of LiCl/ml. were added for induction. As a control, aggregates were kept in a solution containing NaCl at a concentration of 2.57 mg./ml. Both solutions contained Na^{22}. For periods ranging from 0.75 hr. to 5.3 hrs. more Na^{22} was taken up by those aggregates which were in the

TABLE III

Effect of lithium pretreatment on uptake of Na-22 by presumptive epidermis cells

Exp. No.	Treatment		Post-treatment			c.p.m.	No. of agg.	c.p.m. 200 agg.	Difference ± lithium
	Concentration	Hrs.	Conc.	Hrs.	μC. per ml.				
1	300 Li, 515 Na	2.1	450 Na	0.5	1.5	34	110	62	31
	515 Na	2.1	450 Na	0.5	1.5	17	110	31	
2	300 Li, 515 Na	2.1	450 Na	1.0	1.5	45	130	69	24
	515 Na	2.1	450 Na	1.0	1.5	29	130	45	
3	300 Li, 515 Na	2.0	257 Na	1.5	1.5	67	200	67	18
	515 Na	2.0	257 Na	1.5	1.5	49	200	49	

TABLE IV

Effect of lithium during uptake of Na-22 by presumptive epidermis cells

Exp. No.	Treatment		μC. per ml.	c.p.m.	No. of agg.	c.p.m. 200 agg.	Difference ± lithium
	Concentration	Hrs.					
1	300 Li, 257 Na	0.75	1.5	40	95	84	10
	257 Na	0.75	1.5	35	95	74	
2	300 Li, 257 Na	3.0	1.5	38	120	63	33
	257 Na	3.0	1.5	18	120	30	
	300 Li, 257 Na	5.3	1.5	40	115	70	25
	257 Na	5.3	1.5	26	115	45	
3	300 Li, 257 Na	2.0	1.5	50	120	84	29
	257 Na	2.0	1.5	33	120	55	
	300 Li, 257 Na	22.0	1.5	60	120	100	−370
	257 Na	22.0	1.5	282	120	470	
4	300 Li, 257 Na	20.5	1.5	87	125	139	−37
	257 Na	20.5	1.5	110	125	176	
	300 Li, 257 Na	24.5	1.5	85	120	142	−88
	257 Na	24.5	1.5	138	120	230	

solution containing LiCl (Table IV). It is during this period that induction occurs and after 5 hrs. the induction becomes independent of the concentration of NaCl in the culture medium.

For periods ranging from 20 to 24 hours the aggregates in lithium take up less Na^{22} that the controls. The significance of this difference is not apparent but it has no meaning for the induction process, which has occurred during the first 5 hrs. in LiCl. It is clear that the uptake of Na^{22} is higher than controls during and for some time after treatment with LiCl.

Another experiment was designed to determine the length of time, after LiCl treatment, during which the increased uptake could be detected. Thus aggregates were exposed as usual to LiCl and to standard solution. These aggregates then were transferred to standard solution for 1.0 hr. and 2.3 hrs. Aggregates were then exposed to Na^{22} for 30 minutes. Results showed an increased uptake of Na^{22} in LiCl-treated aggregates which had been in standard solution for 1 hr., but no increase over control values in those which had been in standard solution for 2.3 hrs. after LiCl treatment. Therefore the increased permeability produced by LiCl persists for about 1 hr. in standard solution. After this the permeability of LiCl-treated aggregates and control aggregates is about the same as measured by Na^{22} uptake.

In an effort to thrown some light upon the nature of the mechanism of the uptake of Na^{22} by LiCl-treated aggregates, two experiments in which the temperature was varied were carried out. Half the LiCl-treated aggregates were kept at 6° C. and half at 24° C. The Na^{22} uptake of each group was determined during

2.5 hrs. exposure to the isotope. The counts per minute at 6° were 68 and 69, respectively, and at 24°, 164 and 201. These values give a temperature coefficient, Q_{18°, of 2.6.

Discussion

A sequence of cell types beginning with radial nerve and ending with pigment cells can be induced in presumptive epidermis cells (Barth and Barth, 1967, for references). Two kinds of treatments have been found to induce this differentiation sequence: treatment of cells with the lithium ion; treatment with sucrose solutions.

The present experiments demonstrate that lithium and sucrose as inductors have several similarities in manner of action. Induction of new cell types after treatment with either lithium or sucrose is dependent upon the sodium concentration following treatment. In both cases uptake of Na^{22} by cells increases after treatment. The increased permeability indicated by this influx of Na^{22} returns to normal in from 1 to 2 hrs. Induction becomes independent of the sodium concentration in the medium about 2 hrs. after return of cells from inductor solution to standard solution.

A plausible explanation of these data would be that lithium and sucrose increase the permeability of the aggregates to sodium. When returned to a high concentration of sodium, the sodium ion penetrates more rapidly than in untreated controls. This phenomenon completes the process of induction, which becomes independent of the concentration of the sodium ion at about the same time as the permeability decreases to the normal value. This hypothesis, in the case of sucrose as an inductor, throws the entire responsibility for induction inside the cells upon sodium.

When lithium is used as inductor, short treatments with lithium require post-treatment with high concentrations of sodium (*i.e.*, induction is sodium-dependent). Longer treatments with lithium as inductor result in induction even at very low sodium concentrations in the medium used for culture. Therefore, lithium may act inside the cell in a manner similar to sodium, as well as by increasing the permeability of cell surfaces in a manner similar to sucrose treatment.

Further evidence of the action of the lithium ion inside the cell comes from experiments showing synergetic activity of lithium with sodium. For example, 300 mg. LiCl per 100 ml. with no sodium does not induce in 4 hrs. treatment; 300 mg. LiCl plus 257 mg. NaCl does induce in this time; 300 mg. LiCl with no sodium will induce with 20 hrs. exposure. Thus, given time, lithium can act like sodium inside the cell with respect to induction of new cell types.

In the case of sucrose treatment with no ions present, cells must be returned to a medium containing sufficient sodium to complete the induction. There is no evidence as yet that such induced cells show an increase in concentration of sodium over controls, however. This evidence could come only from determination of total sodium in sucrose-treated and untreated cells. Indeed, conceivably the sodium concentration after sucrose treatment could be less than controls, since there is no sodium in the external sucrose solution. The fact that more sodium enters after sucrose treatment may simply be due to a loss of sodium while the aggregates were in sucrose. Upon return to standard solution, cells would find a higher concentration gradient between inside and outside and thus a more rapid penetration of sodium would take place.

This explanation would not hold, however, for the increased penetration of sodium after lithium treatment. Lithium is used in the presence of high sodium and thus loss of sodium from inside would not be expected during treatment. It seems clear that only determination of total sodium concentrations will clarify the situation and reveal whetther or not induction requires an increase in the absolute amount of sodium within the cell.

As regards permeability, we have used the term here although there is some question as to the validity of its use. For example, aggregates treated with sucrose or with lithium are irregular in shape while the controls round up and are spherical at the time of exposure to Na^{22}. Treated aggregates therefore have relatively more surface area, and would be expected to take up more Na^{22}. Our reason for using the phrase "permeability increase" is based upon experiments with uptake of Na^{22} in sucrose. When Ca^{++} is added to sucrose the uptake is reduced to one-sixth the value in sucrose alone. Since Ca^{++} usually reduces permeability of cell membranes we assume that it was acting in this capacity and that we are dealing with the phenomenon of permeability.

The data on temperature-dependence of Na^{22} uptake merit brief comment. A temperature coefficient for $Q_{18°}$ gave a value of only 2.6 for Na^{22} uptake. We can compare this value with the temperature coefficient, $Q_{19°}$ of 8.6, for uptake of ^{14}C-uridine (unpublished). Uridine enters sea urchin eggs by becoming phosphorylated (Piatigorsky and Whiteley, 1965). The results of our temperature studies show that a chemical reaction is involved in the case of uridine uptake by frog gastrula cells. The temperature coefficient for uptake of Na^{22} in frog gastrula cells is very low by comparison and possibly indicates that the penetration is dependent mainly upon diffusion.

SUMMARY

1. The process of induction after sucrose treatment is accompanied by an increased uptake of Na^{22} as compared with the uptake by untreated controls.

2. The process of induction by lithium chloride treatment results in an increased uptake of Na^{22} during the lithium treatment.

3. The process of induction after lithium treatment is accompanied by an increased uptake of Na^{22} as compared with untreated controls.

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NUCLEAR AND CYTOPLASMIC DNA SYNTHESIS IN ADULT AND EMBRYONIC ROTIFERS[1]

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During the embryonic development of rotifers and other Aschelminthes, most cells undergo a fixed number of mitotic divisions. Mitosis and cell division cease early in development; further differentiation thus leads to an adult with a fixed number of nuclei in each organ or tissue. Exceptions to this general rule, due presumably to variations in the number of nuclear divisions in certain organ primordia, are described by Birky and Field (1966). We have used autoradiography with tritiated thymidine to determine whether there is any DNA synthesis in the non-mitotic tissues of older embryos and adults of the rotifer *Asplanchna*. Our results indicate that nuclear DNA synthesis ceases when mitosis ceases, except in the vitellarium, a syncytial organ with 20 to 40 nuclei, analogous to the nurse cells of certain other organisms. In the vitellarium, nuclei are presumably becoming polyploid. We have also detected cytoplasmic DNA synthesis, apparently in mitochondria, in embryos and in adult vitellaria.

Materials and Methods

The studies were carried out on stocks of *Asplanchna brightwelli* from Indiana (inbred clones 5B4S$_7$5, 5B4S$_5$3, and 5B4S$_6$3; *cf.* Birky, 1967a) and Pennsylvania (clone 7B1-1; *cf.* Birky and Field, 1966). The results with the different stocks were identical, and will be considered together. Clones of amictic females, reproducing by diploid parthenogenesis, were reared on *Paramecium aurelia* in infusions of baked lettuce, Cerophyl, or Scottish grass at pH 7.5 and 23° as described by Birky (1964, 1967b).

Thymidine-methyl-H^3 was obtained from New England Nuclear Corporation (specific activity 6.7 c./m*M*) or Schwartz BioResearch Inc. (specific activity 6.0 c./m*M*). The final concentration of H^3-thymidine in the labelling medium was approximately 100 μc./ml. in all experiments. In some experiments adult females were exposed to H^3-thymidine in Dryl's (1959) or Gilbert's (1963) saline solution, for labelling of adult organs *in vivo* and female embryos *in utero*. In other experiments, embryos or adult tissues were dissected out into drops of pseudocoel cavity fluid under oil for labelling *in vitro*. H^3-thymidine in distilled water was then added to the drop; the technique is described in detail by Birky (1967b).

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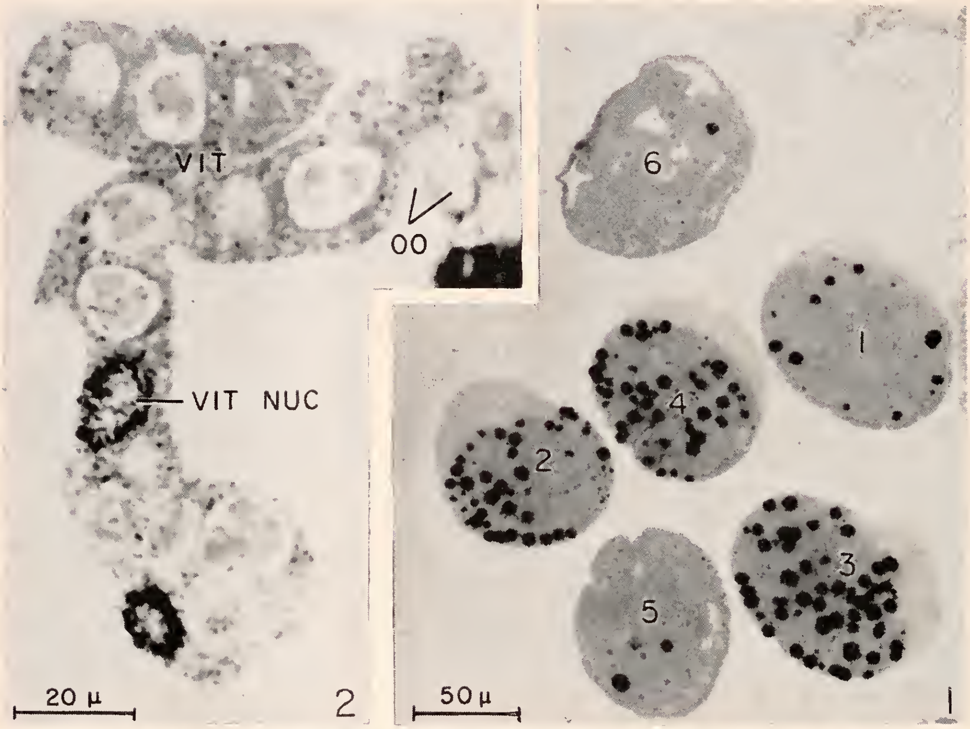
Labelled tissues were processed in several different ways. (1) Adults, embryos, or vitellaria were fixed in 3:1 methanol:acetic acid, squashed in 45% acetic acid, and stained with aceto-orcein after hydrolysis for 10 minutes with 1 *N* HCl at 60°. (2) Adults were fixed in 3:1 ethanol:acetic acid, embedded in paraffin, and sectioned at 4 microns. (3) Adults, embryos, or vitellaria were fixed in glutaraldehyde (2.5% in Dryl's or Gilbert's) or OsO_4 (Palade's fluid, or 2.5% OsO_4 in 0.1 *M* phosphate buffer, pH 7.2–7.4) for one hour, embedded in Epon or methacrylate, and sectioned at $\frac{1}{2}$, 1, or 2 microns. In some cases, squashes or paraffin sections were treated in one of the following ways to test the specificity of the label: (1) deoxyribonuclease (DNase I, Worthington, RNase-free), 0.2 mg./ml. plus 0.003 *M* $MgSO_4 \cdot 7H_2O$ in Michaelis' veronal-acetate buffer without NaCl at pH 6.6 or in 0.01 *M* sodium phosphate buffer at pH 7.5, for 1, 3, or 6 hours at 37°; (2) ribonuclease (RNase, Worthington), 0.2 mg./ml. in Michaelis' buffer, pH 6.6, for 1, 3, or 6 hours at 37°; (3) trypsin (Worthington), 0.2 mg./ml. in 0.05 *M* sodium phosphate buffer, pH 7.4, 25 minutes at 37°. Control slides were treated for the same time and at the same temperature with the appropriate buffer or distilled water. Treated slides were exposd to ice-cold 5% trichloroacetic acid (TCA) for 20 minutes. After thorough washing in water, slides were covered with Kodak AR.10 stripping film or Ilford K.5 liquid emulsion (Caro, 1964) and stored with desiccant in light-tight boxes at 4°. Autoradiographs were usually developed after one to three weeks exposure. Paraffin sections were then stained with Harris hematoxylin; plastic sections were stained with 1% toluidine blue in 1% borax. No grain counts were made; tissues were scored as unlabelled or weakly, moderately, or strongly labelled.

Results

Nuclear DNA synthesis

The development of *Asplanchna* has been described by Lechner (1966), Nachtwey (1925) and others. Complete embryonic development from mature egg to birth requires about 20 hours at 23°. During approximately the first five to six hours, all cells are undergoing rapid mitosis and cell division, with a cell generation time of 15 to 30 minutes. In this early, "mitotic phase" of development, approximately ten cleavages are completed. Few if any nuclei have prominent nucleoli. When embryos of these stages are exposed to H^3-thymidine for 30 minutes or more *in vitro,* all nuclei are heavily labelled as expected (Fig. 1). Preliminary studies indicate an S period of probably less than six minutes duration in the first two cell generations. Nuclei in the S period are labelled when embryos are exposed to H^3-thymidine for as few as 30 seconds.

Mitosis ceases at different times in different tissue anlagen, ranging from about five to seven hours. Nucleoli first appear at this time. Tissues with nucleoli do not appear to contain mitotic figures, suggesting a close correlation between the initial formation of nucleoli and the cessation of mitosis. Moreover, cells with nucleoli are only rarely labelled after short (less than 30 minutes) exposures to H^3-thymidine, suggesting a further correlation between the end of mitosis, nucleolar formation and the end of DNA synthesis. However, our experiments cannot exclude the possibility that some DNA synthesis occurs shortly after the last



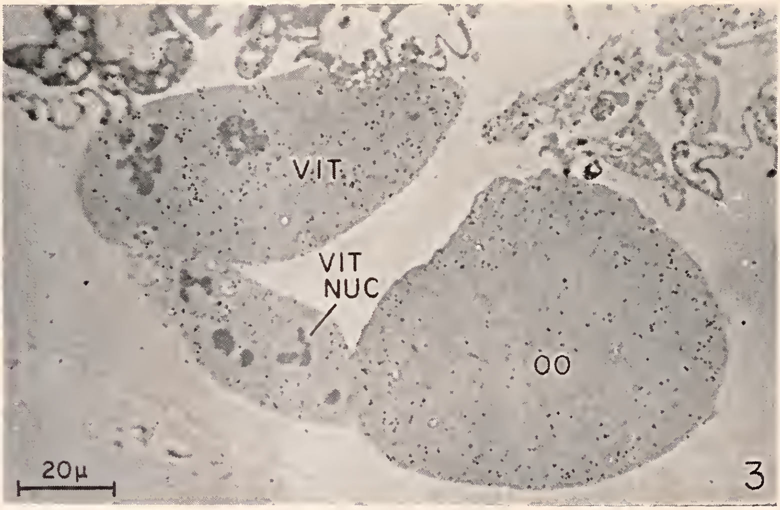


FIGURE 1. *Asplanchna* embryos labelled *in vitro* with H^3-thymidine for 1.5 hours; OsO_4 fixation, 0.5-micron methacrylate section, autoradiograph exposed 21 days. In mitotic phase embryos (1–3), all nuclei are heavily labelled; in transition embryo (4), some nuclei are not labelled, while in post-mitotic embryos (5 and 6), only a few vitellarium nuclei are labelled.

FIGURE 2. Vitellarium of mature female labelled *in vivo* for 4¼ hours; glutaraldehyde and OsO_4 fixation, 2-micron methacrylate section, exposed 14 days. Immature oocytes (OO) are

mitosis, or that nucleoli are formed in some cells shortly before DNA synthesis and mitosis are completed.

In "post-mitotic" embryos and adults, most nuclei have large nucleoli, and no nuclei undergo mitosis. In these older animals, only the nuclei of the vitellarium incorporate H^3-thymidine into DNA (Figs. 1, 2). That this label is indeed in high-molecular weight DNA is indicated by several tests. The label is removed completely or nearly so from paraffin sections or squashes by treatment with DNase but not by the control buffer, ribonuclease, hot 1 *N* HCl, cold 5% TCA, or trypsin (Figs. 4, 5). Adult animals incorporate H^3-thymidine into the vitellarium nuclei at approximately the same rate in the presence of unlabelled uridine at 10 or 100 times the concentration of the thymidine. Finally, identical labelling patterns appear following the use of H^3-thymidine from two different sources and after a variety of different histological procedures.

The labelling of the vitellarium nuclei is highly variable in intensity, making quantitative data almost impossible to obtain. In early post-mitotic embryos, most or all vitellarium nuclei are labelled very heavily after even brief exposures of one hour or less *in vitro* or *in utero*. In contrast, single exposures of nearly mature embryos or of adults result in the labelling of from none to all of the nuclei in a given vitellarium. This variability is found even among animals labelled in the same experiment, under identical conditions. Moreover, the intensity of labelling varies from weak to strong among the different nuclei in a single vitellarium. There is some indication that the frequency of labelled nuclei decreases with age in mature females. The observed variability of labelling could be explained by either of two hypotheses: (1) a variable number of nuclei in adult vitellaria never synthesize DNA, or (2) all vitellarium nuclei carry out DNA synthesis in the adult, but at different times. One experiment argues in favor of the latter hypothesis. New-born females were collected and exposed to H^3-thymidine in four successive pulses, of from 1 to $4\frac{1}{3}$ hours each, at ages zero, 18, 25, and 43 hours. Each pulse was followed by a chase exposure to unlabelled thymidine at 100 times the concentration of the H^3-thymidine. This regime resulted in uniform heavy labelling of all vitellarium nuclei in all females.

We have performed several chase experiments to test the metabolic stability of the labelled DNA in the vitellarium nuclei. Labelled females have been reared in the presence of a 100-fold concentration of cold thymidine for up to 47 hours (about one-half of the life span) without any noticeable decrease in the intensity of the nuclear label or in the number of labelled nuclei. Such experiments indicate that most or all of the DNA synthesized in adult vitellarium nuclei is metabolically stable, at least in young and middle-aged females.

Cytoplasmic DNA synthesis

Animals exposed to H^3-thymidine frequently show incorporation of the radioactive precursor in the cytoplasm (Fig. 3). This cytoplasmic label is largely or

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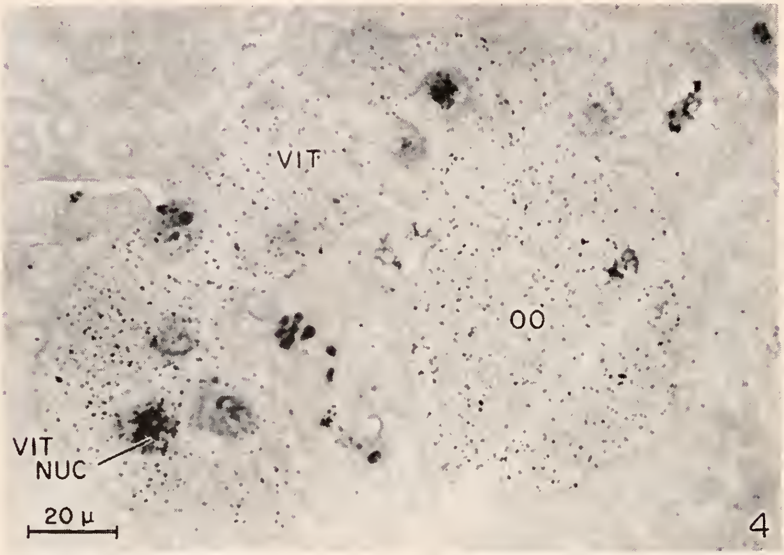
not labelled. Two vitellarium nuclei (VIT NUC) are heavily labelled; the remaining nuclei and the cytoplasm are lightly labelled in the vitellarium (VIT).

FIGURE 3. Adult vitellarium (VIT) with attached, nearly-mature oocyte (OO) labelled as described in Figure 1. Note heavy cytoplasmic label; a few vitellarium nuclei (VIT NUC) are lightly labelled.



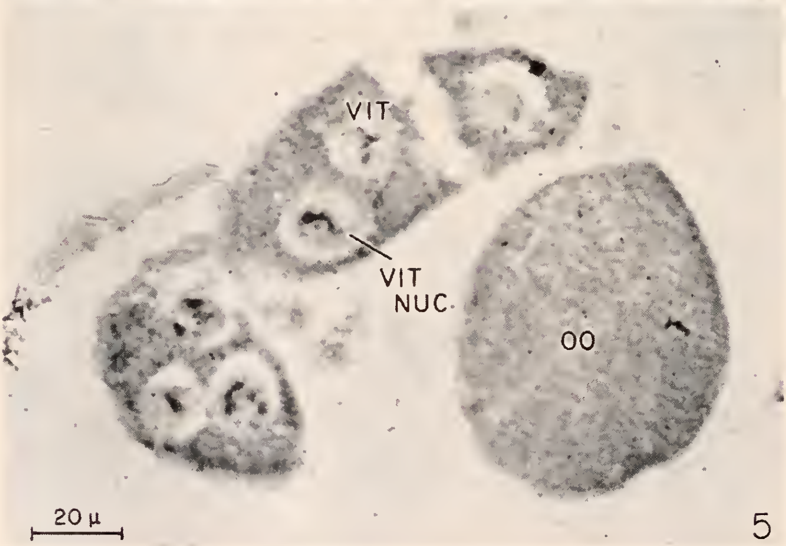


FIGURE 4. Adult vitellarium (VIT) and nearly-mature oocyte (OO) labelled *in vitro* for 30 minutes, fixed in 3:1 ethanol:acetic acid, and embedded in paraffin; the four-micron section was treated with ribonuclease at 0.3 mg./ml. for six hours. Autoradiograph exposed seven days; stained with Harris hematoxylin. Note heavy label over cytoplasm and in some nuclei (VIT NUC); almost complete absence of cytoplasmic basophilia indicates extraction of RNA.

FIGURE 5. Section adjacent to the one in Figure 4, treated with deoxyribonuclease at 0.3 mg./ml. for six hours. Note that cytoplasmic basophilia is retained, but most label has been removed.

entirely in high-molecular weight DNA, as indicated by the same criteria used for the nuclear label (Figs. 4, 5). As a general rule, cytoplasmic labelling is limited to the vitellaria of adults and nearly-mature embryos. However, in some experiments oocytes (Figs. 3, 4) and embryos in cleavage stages are also labelled. As was the case with the vitellarium nuclei, the labelling of cytoplasmic DNA is variable in intensity. When vitellaria are exposed to H^3-thymidine *in vitro* for periods of from 20 minutes to two hours, less than half of the vitellaria show detectable label in a given experiment. Exposure of adult females to H^3-thymidine for from 2 to 23 hours in saline solution results in the labelling of from none to all of the vitellaria in different experiments, with no apparent correlation with length of exposure. Exposure of adult females to H^3-thymidine in the presence of paramecia results in uniform heavy labelling of the cytoplasm, but much of the label is removed by RNase and trypsin. Because of technical difficulties we have not yet been able to obtain any conclusive information about the stability of the cytoplasmic DNA.

It is conceivable that the labelled cytoplasmic DNA is derived in part or entirely from labelled nuclear DNA. It seems more likely, however, that this DNA is synthesized *in situ*. Some vitellaria have heavily labelled nuclei but no detectable cytoplasmic label. Other vitellaria in the same experiment may show cytoplasmic label but little or no nuclear label. For these same reasons, incidentally, a transfer of labelled DNA from the cytoplasm to the nucleus is also unlikely. We are currently using electron microscope autoradiography, as well as light microscope autoradiography under conditions which allow visualization of vitellarium mitochondria, to determine the precise location of the labelled cytoplasmic DNA. Our preliminary results indicate that most or all of the labelled DNA is in mitochondria.

Discussion

Autoradiographic studies such as this one do not distinguish between the replication of DNA molecules, *de novo* synthesis of new molecules without a template, repair of molecules, or the terminal addition of nucleotides onto pre-existing molecules. Nor do they distinguish between DNA which is a functional part of the genome and any other DNA which might be found in the nucleus or cytoplasm. However, in the absence of evidence for more exotic processes, it is reasonable to assume that the incorporation of H^3-thymidine into nuclear DNA represents replication of the genome entirely or in part, even in the non-mitotic vitellarium nuclei of the adult. Indeed, polytenization and polyploidization are common in nurse cells, especially in insects (*cf.* King, 1964; Raven, 1961). In *Asplanchna*, the nurse cells are probably becoming polyploid rather than polytene, as no trace of polytene chromosomes has been seen with either the light or the electron microscope.

A form of limited polyploidy has been reported in amphibian oocytes, in which those DNA cistrons which code for ribosomal RNA are replicated many-fold and the replicas released from the chromosomes to form many independent nucleoli in the nuclear sap (Izawa *et al.*, 1963; Miller, 1964).

In *Asplanchna* there is only one nucleolus in each vitellarium nucleus, and much of the labelled DNA is not closely associated with the nucleolus. Thus if limited polyploidization is involved, it is probably not limited to the nucleolar organizer or ribosomal RNA cistrons. It is conceivable that extra copies of the vitellarium genome, or of selected regions thereof, are required to support rapid synthesis of the cytoplasm which is supplied to the maturing oocyte.

During the post-mitotic phase of rotifer development, cell membranes break down and disappear completely in most tissues, including the vitellarium. This organ is thus a true syncytium during the period of variable nuclear DNA synthesis. Moreover, it is connected to both immature and maturing oocytes by broad cytoplasmic channels. The variability in time and rate of DNA synthesis in the vitellarium nuclei, and the complete absence of DNA synthesis in the oocytes, thus indicate that the cytoplasm of the reproductive organs does not exert control over nuclear DNA synthesis. Similar phenomena have been found in the *Drosophila* egg chamber (Jacob and Sirlin, 1959).

The rotifers may now be added to the growing list of organisms whose mitochondria contain DNA (see Pikó *et al.*, 1967, for references). Cytoplasmic incorporation of H^3-thymidine into DNA has also been reported in another aschelminth, the nematode, *Caenorhabditis briggsae* (Nonnenmacher-Godet and Dougherty, 1964); the precise location of this DNA has, however, not been determined. It appears that DNA can now be considered to be a universal constituent of mitochondria. We are now conducting experiments designed to test the heredity of this DNA during parthenogenetic reproduction.

The authors are grateful to Dr. Daniel Mazia for advice during this project, and to Dr. W. M. Laetsch for the use of his photomicrographic equipment.

SUMMARY

1. DNA synthesis has been studied in adult and embryonic tissues of the rotifer *Asplanchna brightwelli*, using autoradiography with tritiated thymidine.

2. Mitosis and mitotic DNA synthesis cease, and nucleoli are formed, approximately simultaneously at the end of the mitotic phase of embryonic development.

3. In the post-mitotic phase of development and in adult females, the large nuclei of the vitellarium (a nurse cell-like organ) incorporate H^3-thymidine into metabolically-stable DNA, thus presumably becoming polyploid.

4. The DNA synthesis in the different nuclei of the syncytial vitellarium is not synchronous, showing that the cytoplasm does not control nuclear DNA synthesis.

5. Cytoplasmic DNA synthesis also occurs in embryos and in adult vitellaria. Most or all of the cytoplasmic DNA appears to be in the mitochondria.

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THE REGULATION OF CYCLIC REPRODUCTIVE AND FEEDING ACTIVITY IN THE MILKWEED BUG ONCOPELTUS BY TEMPERATURE AND PHOTOPERIOD

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Of the environmental stimuli which have been studied with respect to their effects on insect development and behavior, photoperiod and temperature have received the most attention. Much of this work deals with the effects of these stimuli on polymorphism, growth, diapause, reproduction and general activity (reviews in these areas are given by Lees, 1955; Danilevskii, 1965; de Wilde, 1962; and Birukow, 1966). In recent years, these stimuli have also been considered with reference to their effects on cyclic activities (Williams, 1935; Pittendrigh, 1960; Cloudsley-Thompson, 1961; Harker, 1964; Lewis and Taylor, 1965; and Corbet, 1966); most of these studies deal with either general locomotor activity, flight, or eclosion. Relatively few reports on cyclic reproduction, feeding, or other activities have appeared, exceptions to this being mating and oviposition studies in noctuid moths (Williams, 1936; Larsen, 1943; Shorey, 1964) and oviposition and feeding studies in mosquitoes (Haddow, 1945 *et seq.;* and Gillett, 1962).

Most of the work on cyclic activities in insects has been either purely descriptive or concerned with the analysis of the mechanisms involved, comparatively little attention having been paid to their adaptiveness. Studies where the adaptive value of cyclic activities has been considered are reviewed by Cloudsley-Thompson (1961). Also, there have been few reports where the interaction between two or more cyclic activities has been analyzed. Again, the work on the noctuid moths and mosquitoes represents some progress in this direction, as does, for example, the work of Parker (1962) who described several cyclic behaviors in *Musca domestica.*

This study presents some initial observations on several cyclic activities observed in the large milkweed bug, *Oncopeltus fasciatus,* primary consideration being given to this insect's responses to photoperiod and temperature. A description of the life history of *Oncopeltus* is given elsewhere (Dingle, 1967a, 1967b). An attempt is made to correlate these various cycles and also to assess their possible adaptive significance. This work is part of a continuing program in this laboratory to investigate the behavioral and ecological responses of *Oncopeltus* to its environment as well as the underlying mechanisms involved.

MATERIALS AND METHODS

The insects used in this study were all descended from a culture of *Oncopeltus fasciatus* maintained in the Zoology Department at the University of Iowa. Experimental animals were kept at one of four temperature-photoperiod regimens: 12 hours Light-12 hours Dark, 23° C. (12L-12D 23° C.); 12 hours Light-12 hours

Dark, 27° C. (12L-12D 27° C.); 16 hours Light-8 hours Dark, 23° C. (16L-8D 23° C.); and 16 hours Light-8 hours Dark, 27° C. (16L-8D 27° C.). These animals were descendants of at least two generations maintained at the same temperature-photoperiod regimens. In all cases 20 males and 20 females were maintained together in 17 cm. × 12 cm. × 6 cm. plastic refrigerator boxes with cheese cloth covers and a layer of sand in the bottom. Animals were given dried milkweed seeds for food and had access to water from soaked cotton wool. Loose cotton wool was also supplied for oviposition, and all eggs oviposited were removed prior to hatching. Observations were made with respect to cyclic mating, feeding, and ovipositional activity, and also on cultures placed in continuous light or dark to determine if these activities were free running.

To examine periodic mating activity, 40 imaginal females and 40 imaginal males were collected at eclosion over a 48-hr. period from stocks maintained at the appropriate temperature-photoperiod regimens. Twenty pairs were placed in each of two boxes for each of the four conditions and mating activity was observed for at least 32 days. Any individuals which died during this time were replaced with animals of the same age and sex and which had been maintained under similar conditions. Every two hours, from light on to light off, the number of copulating pairs was recorded; from this the per cent of the total possible pairs mating was computed. After 32 days, the cultures were observed at two-hour intervals during the dark phase of the photoperiod, as well as during the light phase. This was done to determine to what extent mating activity continued in the dark. Observations made during the dark phase lasted approximately one to two minutes and were made under dim red illumination. No effect on activity during the observations was noted and the periodic fluctuations in mating activity during the light phase of the photoperiod remained similar to activity prior to the observations made in the dark phase. Thus it is assumed that these low-intensity, short-duration exposures to light had little or no effect on the mating cycles.

Fluctuations of feeding were recorded from 40 pairs of *Oncopeltus*, 20 pairs per container, in each of the four temperature-photoperiod regimens. In all cases, insects which had reached asymptotic mating frequencies were used. The number of insects feeding at any one observation was determined by simply counting the number of individuals with their proboscises inserted into milkweed seeds. Observations were made every two hours during the light phase of the daily cycle for 10 days and the number of individuals feeding per observation plotted. In the case of the two 12L-12D conditions, an additional observation was added four hours after light off at a time corresponding to that of light off in the 16L-8D photoperiod. These observations were made on the 10 days following the initial observations. A few milkweed seeds were added to the containers after each observation to eliminate possible bias from periodic feeding.

Oviposition cycles were recorded for each condition by removing and counting the number of egg clutches at two-hour intervals during the light phase of the daily cycle. Females in the act of oviposition were not disturbed but were allowed to continue ovipositing and the eggs collected at the next observation. The number of ovipositions per female per hour for each observational period was then calculated. Since oviposition rarely occurred at night, observations were not made in the dark; the number of clutches present at light on was divided to give the average oviposition rate per hour per female during the dark phase. Observations were made and averaged over a ten-day period.

To investigate the possibility of an endogenous control of periodic fluctuations in mating, feeding, and ovipositional behavior in *Oncopeltus,* 60 pairs of insects which had been reared at 16L-8D 23° C. and had reached asymptotic mating levels were transferred to continuous light at the same temperature for five days, and mating, feeding, and ovipositional activity recorded at three-hour intervals. At the end of this period, they were returned to 16L-8D 23° C. and the observations continued for two days. A similar group of 60 pairs was placed in continuous dark for four days and then returned to 16L-8D 23° C., observations being made at three-hour intervals under dim red illumination.

RESULTS

Mating

Mating activity in cultures of sexually mature bugs maintained under the four temperature-photoperiod regimens was cyclic in nature. In all conditions mating activity was at its lowest level of the 24-hour cycle 8 hours after light off. In 12L-12D photoperiods the minimum mating level was maintained during the 4 hours

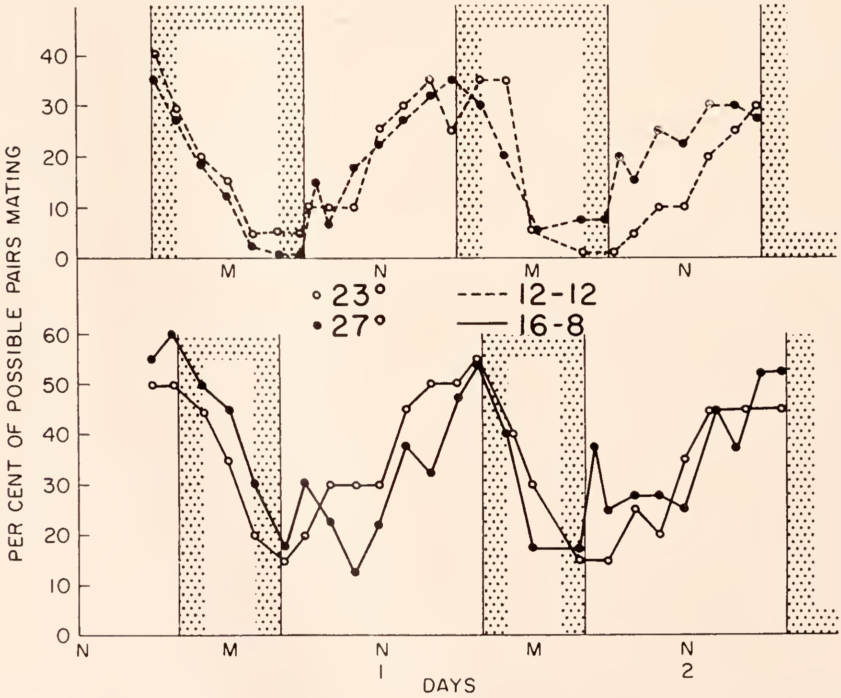

FIGURE 1. Mating activity of 4 cultures of *Oncopeltus fasciatus* reared under 4 different temperature-photoperiod regimens. Activity was plotted over an interval of 2 days. The shaded areas represent dark and the clear areas light phases of the photoperiod. Each culture contained 40 possible mating pairs which were sexually mature.

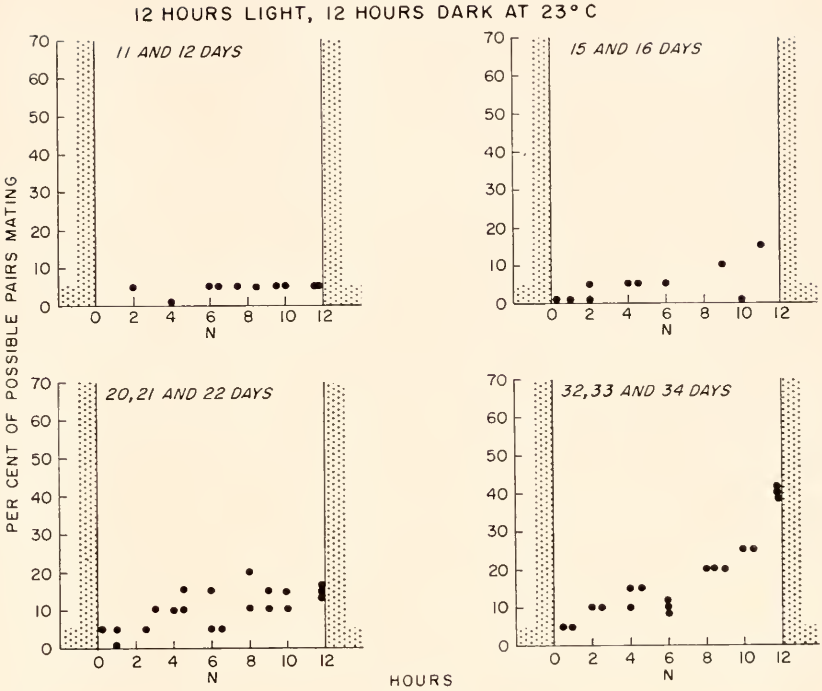

FIGURE 2. The development of the mating cycle in 40 possible mating pairs of *Oncopeltus* maintained at 12L–12D 23° C. First copulation occurred 11 days after adult eclosion, and some cyclic activity is evident after 15–16 days. Asymptotic mating activity had occurred by 32–34 days.

remaining until light on. At light on mating activity began to increase under all regimens, maximum levels being reached at light off, followed by a gradual decrease in mating activity during the dark phase of the photoperiod (Fig. 1).

First copulations were observed from 4 to 11 days following adult eclosion depending on conditions. Under high temperature and long photoperiod, 16L-8D 27° C., first matings occurred at 4 days. Under low temperature and short photoperiod, 12L-12D 23° C., first matings were delayed until 11 days after adult eclosion. In cultures reared under high temperature and short photoperiod or low temperature and long photoperiod the time to first mating was of intermediate duration, 8 and 9 days, respectively (Figs. 2–6).

Temperature and photoperiod also determined the maximum mating levels reached under a given regimen and how quickly these asymptotic mating levels were achieved. Photoperiod alone determined the asymptotic mating levels reached within a regimen. In 16L-8D, at both 23° C. and 27° C. a maximum of 65–70% of the possible pairs was observed mating. In 12L-12D, at either 23° or 27° C. a maximum of only 40% of the possible pairs was observed mating. Observations made on older cultures of *Oncopeltus* confirmed that maximum mating levels achieved during the first 30 days under these conditions were indeed asymptotic. There was no overlap between photoperiods.

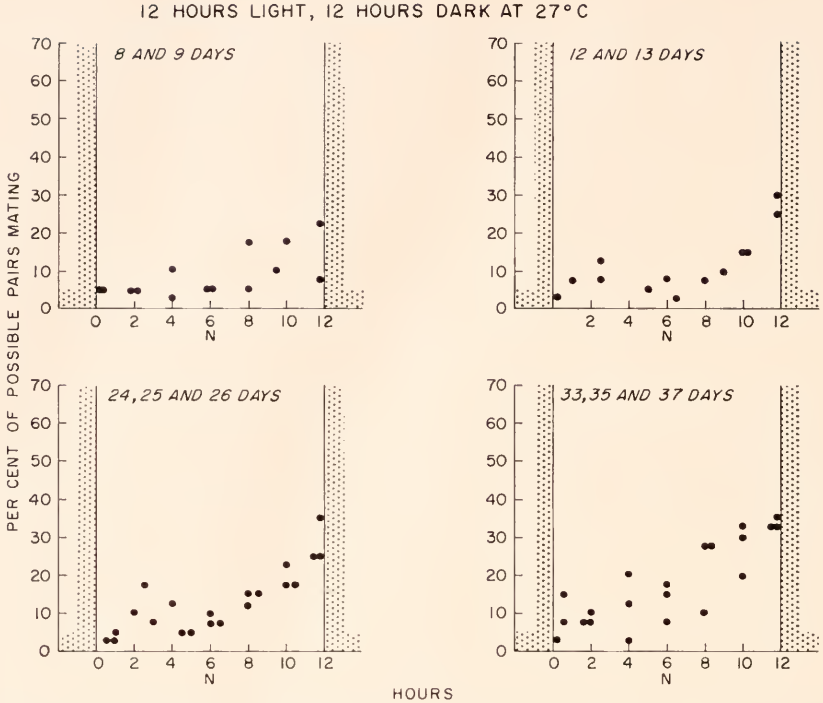

FIGURE 3. The development of the mating cycle in 40 possible mating pairs of *Oncopeltus* maintained at 12L–12D 27° C. First copulations occurred 8 days after adult eclosion at which time some cyclic activity was apparent. Asymptotic mating levels had already been achieved by days 24–26.

Temperature, as well as photoperiod, did appear to affect the rate at which asymptotic mating levels were achieved. At 16L-8D 27° it took only four days to reach asymptote once mating was begun and at 12L-12D 27° C. 75% of the asymptotic mating level was achieved three days from the onset of mating and full asymptote after 12 days. At 23° C., on the other hand, asymptotic mating levels were reached more slowly, taking 16 days from onset of mating under a 16L-8D photoperiod and 21 days under a 12L-12D photoperiod. Figures 2–5 illustrate the development of mating cycles under the four regimens used. Figure 6 summarizes the development of mating activity under the four regimens, giving the maximum percentage of the possible pairs mating on a given day following adult eclosion. In all cases the maxima so plotted occurred not more than 2 hours prior to light off.

Feeding and oviposition

A striking similarity is noted between the curves derived for periodic mating and feeding. Feeding was at a low level at light on, with perhaps a small increase in activity shortly thereafter. It declined to minimum levels eight hours after light on and then increased rapidly to reach maximum values at light off. This was

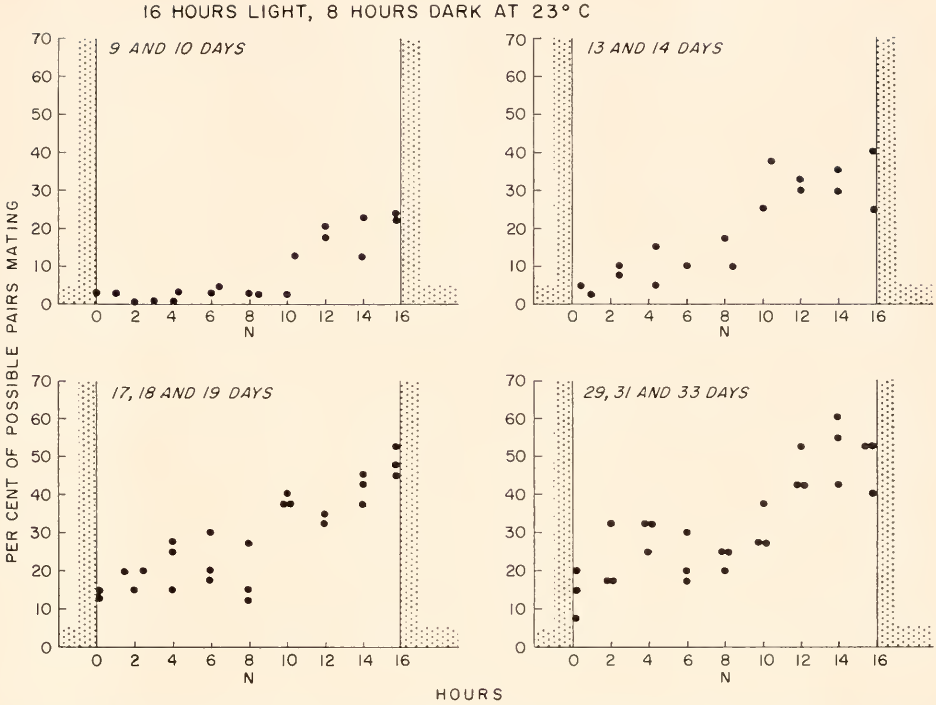

FIGURE 4. The development of the mating cycle in 40 possible mating pairs of *Oncopeltus* maintained at 16L-8D 23° C. First copulations occurred 9 days after adult eclosion at which time cyclic activity was evident. Asymptotic mating levels had been achieved by days 29–33.

true for all four conditions. At both temperatures the 16L-8D bugs achieved a higher percentage of feeding than did those at 12L-12D. In the 12L-12D regimens, however, feeding was maintained at maximum levels for at least four hours following light off (Fig. 7A). Females on 12L-12D or 16L-8D almost always oviposit during the light phase of the photoperiod, with little or no egg-laying occurring after light off. Under 16L-8D 23° C., 16L-8D 27° C., and 12L-12D 27° C. regimens oviposition reached a peak 8 hours after light on with values from 0.06 to 0.075 ovipositions per female per hour. Although the rate of oviposition was much lower at 12L-12D 23° C., reaching a maximum value of 0.02 ovipositions per female per hour, the peak activity still occurred 8 hours after light on (Fig. 7B).

Free running rhythms

When *Oncopeltus* maintained at 16L-8D 23° C. and at asymptotic mating activity were placed in continuous light for five days, mating did not begin to decline as usual 16 hours after light onset, but was maintained at high levels for the next six hours before a slight dip in activity was noted. During the next five days of continuous light, mating activity did not fall to normal minima. The first three cycles in constant light had a period of mating activity of 21 hours; a

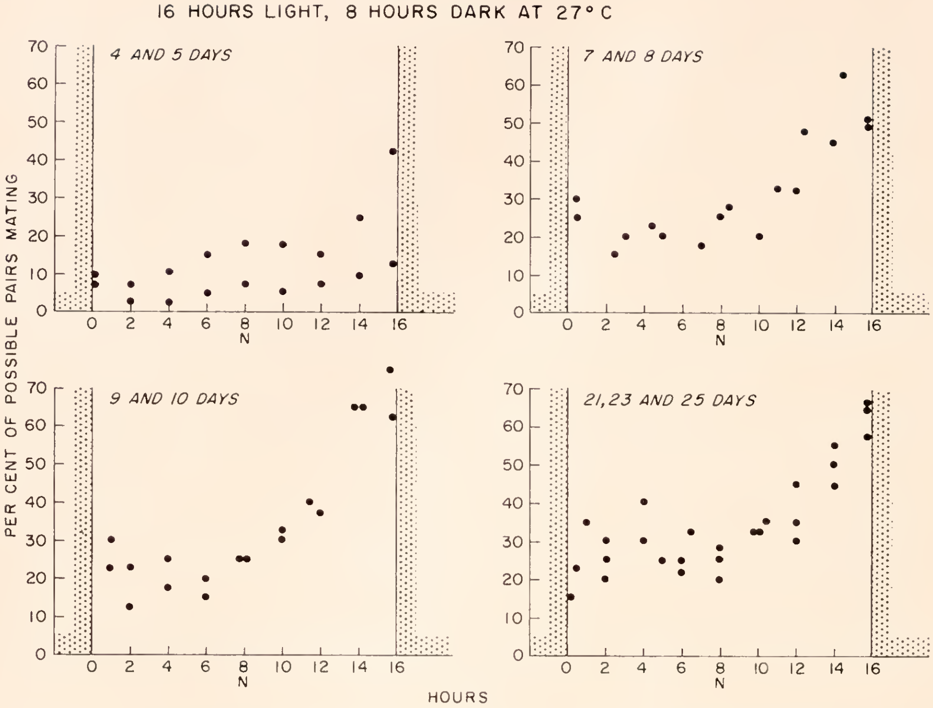

FIGURE 5. The development of the mating cycle in 40 possible mating pairs of *Oncopeltus* maintained at 16L–8D 27° C. First copulations occurred 4 days after adult eclosion at which time cyclic activity was present. Near asymptotic mating levels were achieved by 7–8 days and full asymptote by days 9–10.

fourth cycle had a period of 27 hours. When a similar group of 60 pairs was placed in continuous dark for four days, mating activity was depressed during the entire four days. There did, however, appear three peaks of mating activity, the first two cycles having a period of 21 hours and the third cycle a period of 24 hours. After three to four days in either continuous light or dark, mating activity became aperiodic (Fig. 8A).

Feeding, in general, was maintained at a slightly higher level under continuous light than under continuous dark. One difference noted between mating and feeding activity under continuous light was that feeding activity fell from maximum levels reached 16 hours after light onset to minimum values 8 hours later. One cycle of feeding activity under continuous light was recorded, having a period of 21 hours, after which cyclic activity apparently broke down. In continuous dark, no cyclic feeding was observed, although upon return to 16L–8D a peak of activity was reached 9 hours after light onset and at the normal time of 16 hours after light onset during the second 24-hour cycle (Fig. 8B).

Oviposition cycles were the most persistent of the three measured activities under both constant light and dark. Under continuous light there were periodic fluctuations in oviposition during the entire five-day period. The periods of the

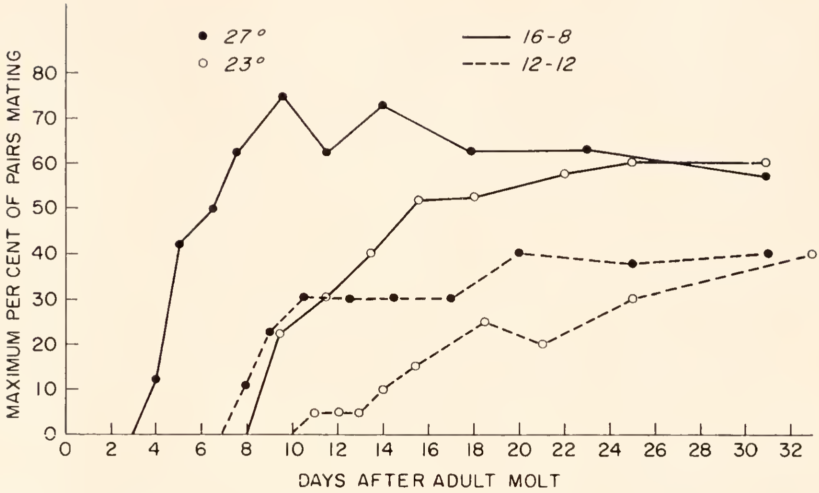

FIGURE 6. Maximum mating levels achieved by 4 groups of *Oncopeltus fasciatus* reared under 4 different temperature-photoperiod regimens. The maximum per cent possible pairs mating on a given day following the adult molt was plotted for each group and in every case the maximum occurred within 2 hours of light off. Note that photoperiod alone determines the maximum level of mating achieved within a regimen while both photoperiod and temperature determine the time to first mating and also the time to asymptotic mating levels.

five cycles were in order, 18, 21, 21, 24, and 27 hours. In continuous dark, the first peak of egg-laying came 21 hours after the preceding one, the remaining three cycles which occurred being somewhat variable, having periods of 21 to 27 hours. Under both conditions the amplitude of the fluctuations in oviposition were depressed, although no change in the overall number of clutches oviposited per 24 hours occurred (Fig. 8C).

DISCUSSION

In the laboratory, feeding and reproductive activities of *Oncopeltus fasciatus* occurred in daily cycles governed by the environmental regimens under which the animals were maintained. In these experiments only temperature and photoperiod were varied systematically, and within each regimen photoperiod alone provided possible "clues" (Cloudsley-Thompson, 1952) or "Zeitgeber" (Aschoff, 1954) which could synchronize endogenous rhythms or determine cycles exogenously. The results indicate that photoperiod can control cyclic mating, feeding, and oviposition in *Oncopeltus.*

The patterns of these activities are summarized in Figure 9. Note first that oviposition cycles are distinctly out of phase with mating and feeding. In both 12L-12D and 16L-8D egg-laying activity reached a peak 8 hours after light on while in the case of the latter two functions a peak was not attained until at least 12 hours. The fact that oviposition remains fixed with respect to light on indicates that this is the Zeitgeber that triggers or synchronizes egg-laying.

Feeding cycles under both photoperiods were identical for the first 12 hours after light on; at light on, feeding was at near minimal levels, but then showed a

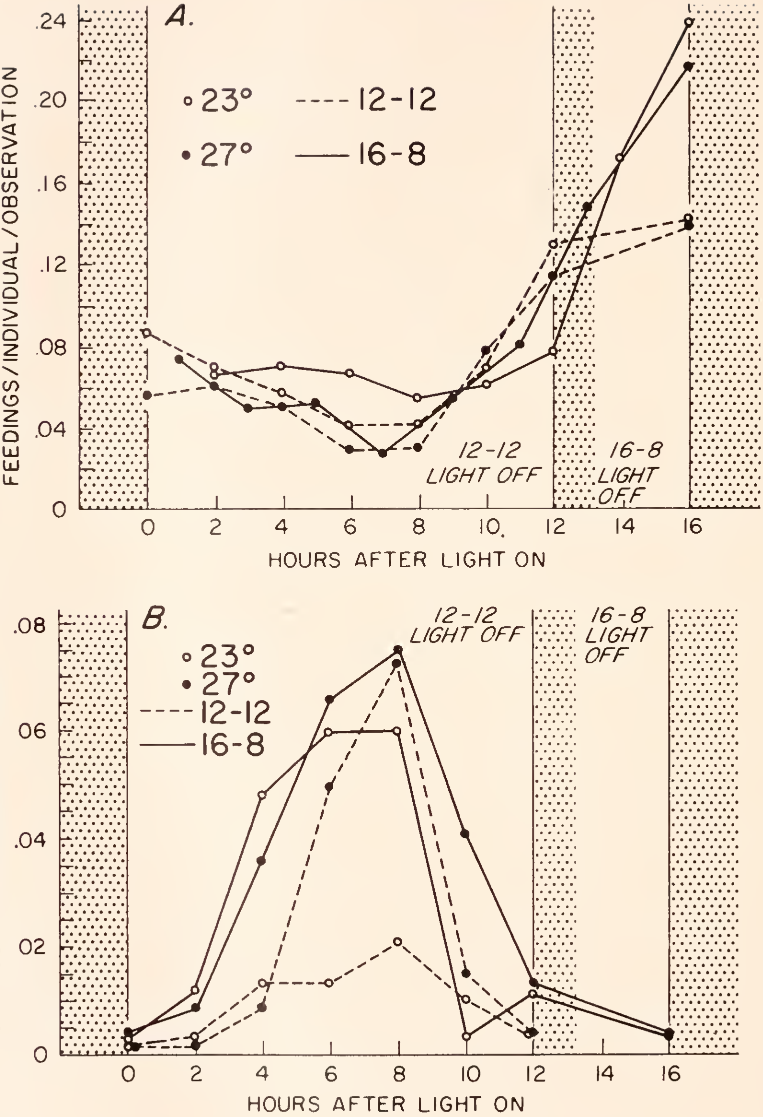

FIGURE 7. A. Feeding. B. Oviposition. Feeding and oviposition activity observed under 4 temperature-photoperiod regimens in 40 pairs of *Oncopeltus* per condition. Points represent the means of values taken over a 10-day period. Note that in all groups oviposition reached maximum 8 hours after light on while feeding reached maximum 16 hours after light on (4 hours after light off for 12L–12D regimens).

small transitory rise lasting about two hours. This brief early burst is presumably associated with an increase in locomotor activity immediately following light on (Caldwell and Dingle, unpublished observations) and the resulting increased probability of locating food. Eight hours after light on feeding fell to the lowest

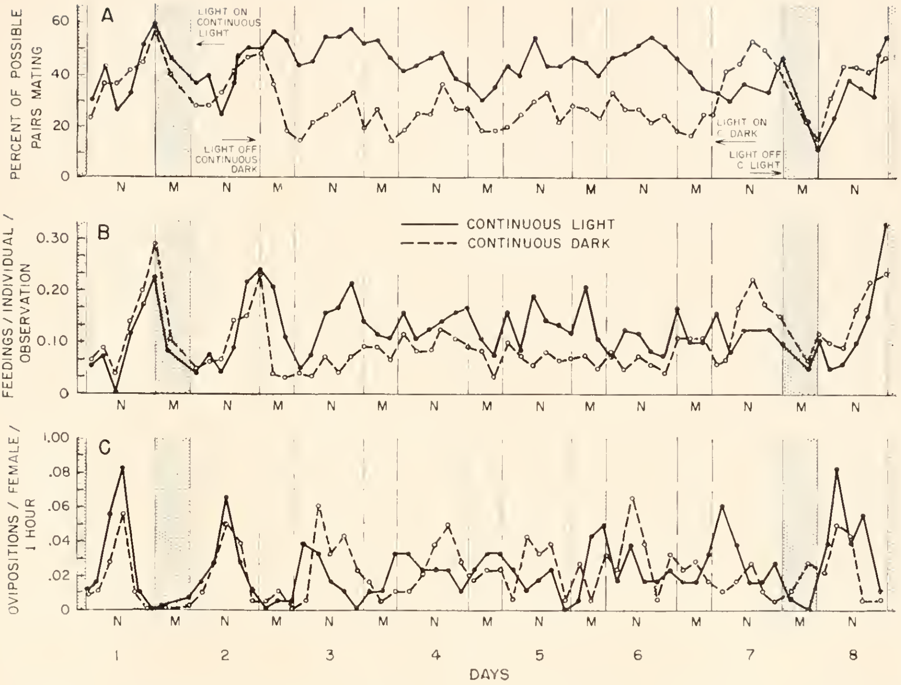

FIGURE 8. Activities observed in continuous light and dark. A. Mating. B. Feeding. C. Oviposition. Animals placed in constant light or dark at 23° C. were reared at 16L-8D 23° C. Broken vertical lines represent times that light on and off would have normally occurred. Note that in all cases (except Feeding, constant dark) at least one cycle of activity is observed after the onset of constant conditions.

levels recorded during the cycle, and then climbed rapidly until light off. During the four hours following light off, feeding in the 12L-12D cultures remained at or slightly increased above the levels reached at light off (Fig. 7A), whereas it began to fall at light off under 16L-8D (Fig. 8B). The difference in the maxima of the cycles under the two photoperiods is apparently due to the time at which light off occurs; in either case the onset of darkness damps further increases. This results in more feeding under 16L-8D than 12L-12D photoperiods. While growth, maturation, and reproduction could be affected indirectly *via* varying food intake as a result of differing photoperiods, feeding activity does not necessarily reflect nutrient intake (Feir and Beck, 1963).

Mating cycles under the two photoperiods were not in phase; in either condition, however, they were at minimum levels at light on. The initial increase in activity may be due to increased locomotion as in the feeding cycle, leading, in this case, to an enhanced probability of finding a mate. In the 16L-8D photoperiod, mating declined to minimal levels six hours after light on. Under 12L-12D, on the other hand, there was only a reduced rate of increase for a short period in the possible pairs mating. By six hours after light on, mating was the same under both photoperiods and continued to rise until light off. At light off, it began to decline and

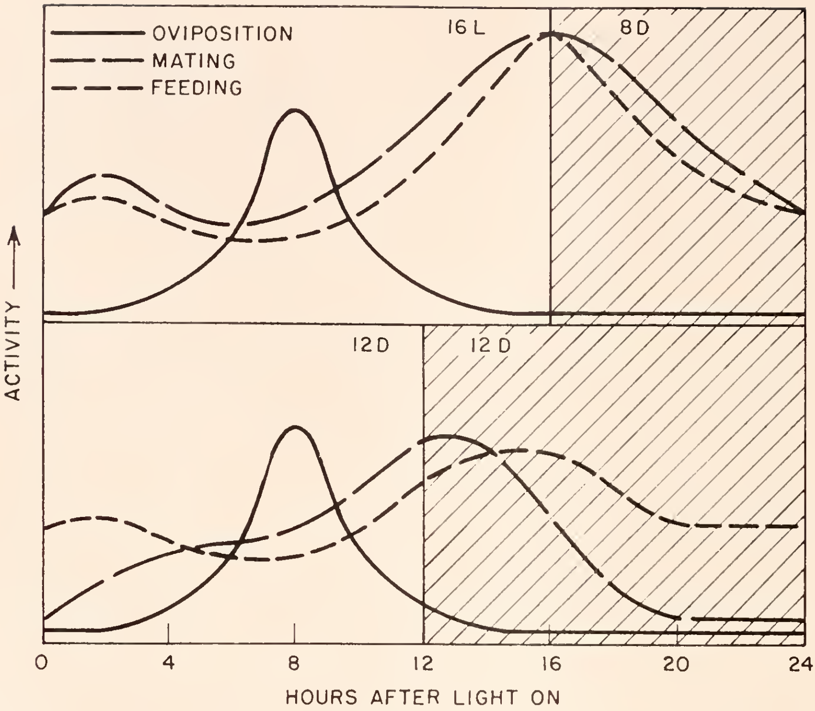

FIGURE 9. Interaction of mating, feeding, and oviposition cycles in *Oncopeltus fasciatus* under 12L–12D and 16L–8D photoperiods. Activity is represented in arbitrary units. Curves were constructed from data taken from cultures of sexually mature insects at both 23° and 27° C. (temperature has no effect on these cyclic activities over this range). Note that under both photoperiods all three activity cycles appear to be similar for the first 12 hours after light on and that dark suppresses further increases in mating and feeding.

reached minimal levels eight hours later. It appears that the duration of the light period determines the level of maximum activity, long days producing high levels, while the duration of the dark period determines the minima, long nights producing low levels. Light off shuts down mating and on a short day does so before it has reached its peak; the relatively short 8-hour night phase following a 16-hour day prevents a fall to near zero. Direct control by photoperiod is probably an oversimplification, however, for the cycles of animals reared in 16L-8D do not fall below normal minima, approximately 20% of possible pairs mating, even when the cultures are placed in continuous dark (Fig. 8A), nor is there much overshoot when in continuous light. Maximum amplitude thus seems to be reached on a 16-hour day. Factors other than direct stimulation by light or inhibition by dark therefore probably also affect mating activity.

In addition, continuous light or dark influenced the expression of all three activities measured (Fig. 8). In each instance, both in continuous light and continuous dark (except continuous dark, feeding) one cycle of activity was observed during the first 21 to 24 hours of constant conditions; further cyclic activity persisted in some cases, *e.g.*, oviposition, during the entire five-day period.

In general, the free running rhythms had initial periods of approximately 21 hours in continuous light. (Since observations were made at three-hour intervals, it is not possible to make a more precise statement as to the actual period length.) In the case of both mating and oviposition, the period was observed to lengthen after three days to 24 hours. In continuous dark, the free running rhythms were less persistent, although the first cycle also had a period of 21 hours. The periods of later cycles were more erratic, but in general were 24 hours or longer. Beck *et al.* (1958) and Feir and Beck (1963) observed the occurrence of a feeding rhythm in *Oncopeltus* fifth instar numphs under constant conditions (continuous dark interrupted one second each minute by a flash of light), indicating that an endogenous feeding rhythm is present even before the imaginal molt. Experiments are presently underway to define more precisely the free running rhythms found in *Oncopeltus* and to determine the role they may play in the activity cycles reported here.

There is in any case integration of the three cycles. Towards the end of the daylight hours, both feeding and mating reach a peak, but these two activities are not in conflict since *Oncopeltus* is able to mate and feed simultaneously. During the early afternoon hours, females oviposit; feeding and mating have not yet begun to increase so no conflict occurs. It may also be of interest to note that a review of the data from *Oncopeltus* flight tests made to determine the potential for migration (see Dingle, 1965 for a summary of procedures) indicates that significantly more bugs flew ("migrated") in the early afternoon, the same time that oviposition is at a peak. Migration, however, generally occurs pre-reproductively in *Oncopeltus* (Dingle, 1965 *et seq.*) and therefore would not interfere with either oviposition which has not yet begun or feeding which occurs in the evening. Some mating may occur at the same age as peak flight activity, but again would not be in conflict with flight since it also occurs mostly in the evening.

A causal relationship between the various cyclic activities can not at this time be specified. Activity cycles may be due to one underlying factor or an interaction of several discrete physiological events. Feeding cycles occur during the first few days after the imaginal molt and may be present during juvenile stages (Feir and Beck, 1963), while mating is initiated 4 to 10 days and oviposition 8 to 15 days following adult eclosion. This would seem to indicate that feeding and mating cycles are independent of the oviposition cycle, although again, the same ultimate physiological mechanism may underlie all three activities.

Although they were not investigated in this study, general locomotor activity and a possible cyclic pheromone production may influence feeding, mating and oviposition. Cyclic locomotor activity was not quantified, but general differences in levels of activity were noted. The bugs were more active in light than dark and also showed a marked increase in activity at light on. As mentioned above, such an increase in locomotion at light on could be related to corresponding bursts in feeding and mating. Experiments are now in progress to analyze the relationship between general activity and the cyclic behaviors described above.

Pheromonal control of mating is suggested by the observation that males attempt to copulate with females of already mating pairs and that introduction of a copulating pair into a culture triggers a burst of mating. Cyclic pheromonal production similar to that postulated in several Lepidoptera (Ouye *et al.*, 1965;

Shorey and Gaston, 1965) could exert control over cyclic mating in *Oncopeltus* and experiments are also in progress to explore this possibility.

The adaptive significance of cyclic activities in insects is difficult to assess and relatively few attempts have been made to determine their ecological importance. With respect to *Oncopeltus*, studies made in August, 1966, indicate that, as in the laboratory, a mating cycle exists in the field with maximum activity occurring in the early evening. Furthermore, a majority of the mating pairs were found on the seed pods of milkweed plants (the major source of food for *Oncopeltus* during this time of year) and many of these animals were feeding. Localization of feeding sites and the ability to mate while feeding may serve to bring pairs together, especially if a peak of activity occurs during one part of the day. Such a mechanism might be particularly adaptive in the early summer when population densities are low and feeding sites relatively scarce. Why mating and feeding should occur primarily in the early evening remains obscure except as a result of possible interactions with other activities. As mentioned above, flight occurs principally in the early afternoon. This corresponds with maximum daily temperatures and may be advantageous since high temperatures lower the threshold for flight. Suppression of mating and feeding during this period would assure maximum opportunity for flight to occur. A similar situation seems to occur in *Leptohylimyia coarctata,* a wheat bulb fly, in which an endogenous oviposition rhythm occurs with maximum activity two hours before dark while flight occurs in the early morning and later evening (Long, 1958). As noted, however, oviposition and migration flights generally do not overlap in the life-cycle of *Oncopeltus* females so that the occurrence of oviposition in the early afternoon would appear not to be related to flight activity. Environmental conditions, however, may promote oviposition at this time. For whatever reason, there seems to be some selective advantage in laying eggs in the afternoon since several other Hemiptera occupying somewhat similar niches, *e.g., Anasa tristis* (Beard, 1940), *Nysius huttoni* (Eyles, 1963) and *Euschistus conspersus* (Hunter and Leigh, 1965), have been reported to have oviposition cycles with peak activity occurring at that time.

Aside from determining the timing and amplitude of the various activity cycles in *Oncopeltus*, photoperiod, in combination with temperature, determines the time to onset of reproduction. Andre (1934) noted decreased time from the imaginal molt to first reproduction in *Oncopeltus* with an increase in temperature. Similar results were observed over the temperature range used in this study. But in addition, lengthened photoperiod also shortens the time to first copulation. Thus higher temperatures and longer photoperiods caused a rapid onset of reproductive activity while lower temperatures and shorter photoperiods delay reproduction. Such responses may be adaptive for a migrant such as *Oncopeltus* since delays in reproduction allow time for more migratory flights to occur (Dingle, 1967b). Thus in the early spring or late fall with short photoperiods and lower temperatures, there will be time for migratory flights; in the summer under longer photoperiods and higher temperatures reproduction will begin earlier with fewer and shorter flights occurring. The dependence of the prereproductive period on the photoperiod as well as temperature affords some protection from short periods of unseasonably high or low temperature, assuring that migration will occur in the spring and fall while reproduction continues during the summer. Also, early reproduction leads to more rapid population growth (Cole, 1954), an advantage for a migrant colonizer invading an empty habitat as *Oncopeltus* does in the late spring or early summer in the central and northern United States (Dingle, 1967b).

Blatchley (1926) reported that *Oncopeltus* may spend the winter in the southwestern United States in a state of quiescence, becoming active only on warm days and evidently not reproducing. It would be easy to extrapolate the photoperiodic-temperature response of *Oncopeltus* (Fig. 6) to a point where at short photoperiod and low temperature little or no reproductive development would occur and the adult would enter a non-reproductive period as occurs, for example, in *Plutella maculipennis* (Harcourt and Cass, 1966) and *Lygus hesperus* (Leigh, 1966). Such a delay in reproduction in response to short photoperiod and low temperature, as is found in *Oncopeltus,* probably represents an incipient diapause and an intermediate step in the evolution of adult reproductive diapause (Harcourt and Cass, 1966; Dingle, 1967b).

This paper has presented some initial observations on the responses of *Oncopeltus fasciatus* to two environmental stimuli, photoperiod and temperature. It is hoped that by accumulating a more complete knowledge of the repertoire of responses by *Oncopeltus* to the environment, such as possible locomotor activity cycles, reproductive responses to wider ranges of environmental conditions, social interactions, etc., a more integrated view of the behavior and ecology of *Oncopeltus* may be achieved, including some insight into the basic mechanisms underlying cyclic activities (Pittendrigh and Minis, 1964; Adkisson, 1966; Lees, 1966).

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SUMMARY

1. Mating, feeding, and oviposition cycles were observed in the large milkweed bug, *Oncopeltus fasciatus*, under four regimens of varying temperatures and 24-hour photoperiods.

2. Mating and feeding activity reached maximum at the end of the light phase while oviposition occurred 8 hours after light on.

3. When placed in continuous light or dark, cyclic activity of all three behaviors persisted for at least one cycle and in the case of oviposition for five cycles. In general, the period of the first one or two cycles for all three activities, in both continuous light and dark, was approximately 21 hours; subsequent cycles, if present, had periods of 24 hours or longer.

4. Photoperiod was found to affect the maximum mating and feeding activity which occurred during the day. Long photoperiods promoted higher levels of mating and feeding than did short photoperiods.

5. The time from eclosion to first mating was photoperiod- as well as temperature-dependent; longer photoperiods and higher temperatures shortened the time to first mating.

6. The interaction of the various cyclic activities was discussed in reference to the ecology of *Oncopeltus.* The hypothesis was proposed that the occurrence of

most mating and feeding in the early evening would allow maximum opportunity for oviposition or migratory flight, which reach peak activity earlier in the day, to occur.

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AN INVESTIGATION OF FACTOR S, A NEUROMUSCULAR EXCITATORY SUBSTANCE FROM INSECTS AND CRUSTACEA [1,2]

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The neuromuscular function in vertebrates and in a number of invertebrates depends upon a cholinergic system. Such, however, does not appear to be true of arthropods. Repeated attempts to demonstrate cholinesterase in insect muscle have failed (Wigglesworth, 1958 and Colhoun, 1958), and such pharmacological agents as acetylcholine and paraxon and atropine have had no effect on neuromuscular transmission (Harlow, 1958; Katz, 1936; Usherwood, 1963). However, the ultrastructure of the nerve-muscle junction in several insects does suggest a chemical type of transmission (Smith and Treherne, 1963).

Several recent reports suggest that L-glutamic acid serves as an excitatory transmitter at the myoneural junction in both Crustacea and insects (Takeuchi and Takeuchi, 1964; Ozeki *et al.*, 1966; Kerkut *et al.*, 1965; and Usherwood, 1966). However, the possibility of a second substance in myoneural function is suggested by the work of Van der Kloot (1960). He found a substance in the crayfish, *Cambarus clarkii* (Girard), which stimulated the contraction of the closer muscle of the claw. This substance, called Factor S, was detected in perfusates from stimulated claws but not in perfusates from unstimulated claws. Van der Kloot extracted Factor S by the aluminum hydroxide precipitation procedure of Von Euler (1948), and he noted that this substance had several chemical properties in common with an unidentified catechol-4 isolated by Ostlund in 1954 from a number of invertebrate species.

We used the Von Euler extraction procedure on several species of arthropods in an attempt to confirm the findings of Van der Kloot and Ostlund. We have obtained a substance that has a number of properties in common with both Factor S and catechol-4.

MATERIALS AND METHODS

1. *Extraction procedure*

All equipment and solutions used in the initial deproteinization were brought to 4° C. Whole animals or specific tissues were weighed and ground in a blender with 3 volumes of 8% trichloroacetic acid for five minutes. The homogenates were

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[1] Mention of proprietary products does not necessarily imply endorsement by the United States Department of Agriculture.

[2] Presented in part before American Society of Zoologists, College Park, Maryland, August 17, 1966.

allowed to stand for ½ hour so the amines could be extracted before centrifugation at 4500 rpm for 10 minutes. The precipitated protein was discarded, and the supernatant was passed through a coarse grade, sintered glass funnel under a positive nitrogen pressure to remove non-sedimenting cuticular fragments. At this point, the supernatant was extracted with ether (1 : 1 ratio) to remove excess trichloroacetic acid and interfering lipids. A flocculate of aluminum hydroxide was then formed in the supernatant by first adding 1 ml. of 20% aluminum sulfate for every 100 ml. of solution and then adding 0.5 N sodium hydroxide (with constant stirring) until a pH of 7.5 was reached. The aluminum hydroxide was removed from the solution by centrifugation at 4500 rpm for 10 minutes. The precipitate was then dissolved in a minimum amount of N sulfuric acid. Sufficient 0.5 N sodium hydroxide was added to bring the solution to a pH of 2.8. Extraneous salts were removed from the solution by adding 4 volumes of ethanol. This mixture was allowed to stand for 15 hours at 4° C. to complete the precipitation process. The salts were removed by centrifugation, and the resulting supernatant was taken to dryness *in vacuo* at 40° C. Further desalting of the residue was usually necessary and was accomplished by the addition of 100 ml. of acetone. The mixture was then allowed to stand on ice for 45 minutes to complete the process, the salts were discarded, and the supernatant was again taken up in 10 ml. of 0.5 *M* sodium phosphate buffer (pH 6.5) and placed on a cation exchange resin.

2. *Chromatographic purification of Factor S*

An Amberlite IRC 50 cation exchange resin was prepared in the sodium form described by Bergström and Hansson (1950). This ion exchanger separated the catecholamines from organic acids, sugars, and natural pigments occurring in crude extracts. The column containing the crude extract was eluted with 75 ml. of 0.2% sodium chloride and then by 75 ml. of 1 N HCl. The N HCl fraction from the column was taken to dryness, and the residue was dissolved in 0.2 ml. of ethanol. The eluate was then spotted on Whatman No. 1 filter paper with a micropipet, and the solvent was removed by a stream of nitrogen. The spotted papers were chromatographed in a phenol-0.1 N HCl system (1 : 1) in a descending system for 18 hours at 24° C. ± 1°.

Initially, the position of Factor S was determined by bioassay; later, spray reagents such as potassium ferricyanide and naphtho-quinone-4-sulfonate were used. Generally, the active area from the phenol system was eluted and rechromatographed in a butanol-acetic acid-water system (4 : 1 : 5) before assay.

PHYSIOLOGICAL PREPARATIONS

1. *Isolated ventral nerve cord*

Adult male American cockroaches, *Periplaneta americana* (L.), were decapitated, opened from the dorsal surface, and pinned ventral side down on a piece of cork. The thoracic portion of the nerve cord was removed and placed on recording electrodes. The preparation was perfused with the saline solution described by Twarog and Roeder (1957). The electrodes were connected to the push-pull input of a Grass P-5 preamplifier. A Hewlett-Packard oscilloscope was employed to monitor endogenous activity. The most frequently observed response in untreated preparation consisted of a steady but arrhythmic background of action potentials that ranged in amplitude between 30 and 100 μv. These potentials ranged from 50 to 1000 cps in frequency (Fig. 2a). This level of activity remained constant for several hours. For comparison purposes, a preparation of the abdominal nerve cord of the crayfish was used. Figure 2f shows normal activity on such an isolated nerve cord. With such preparations, Van Harreveld's solution was used to perfuse the isolated cord while it was in contact with the electrodes.

2. *Motor nerve preparation*

An *in situ* preparation of the cockroach motor nerves was used to bioassay the substance. Adult males were decapitated, slit along the dorsal midline from the last abdominal segment through the prothoracic segment, and pinned ventral side down on a cork platform. The region surrounding the metathoracic ganglion was exposed, and all nerves leading to the ganglion except the abdominal and forward thoracic connectives were severed. Fine platinum electrodes were placed underneath the severed ends of nerves 5 or 6. The activity of such preparations was observed for 10 to 15 minutes until activity was considered steady. Samples of Factor S were then placed on the ganglion with a micropipet.

3. *Nerve-muscle preparation*

Male cockroaches were decapitated and pinned dorsal side down through the prothoracic and 6th abdominal segments to a paraffin-filled petri dish. The sternal sclerites of the metathoracic segment were removed to expose the ganglion, and the fifth was exposed more extensively by removing the cuticular membrane running between the coxa and the sternite. The anterior of the coxa was then rotated 90° to bring it into a ventrally upright position. The coxa was held in this position with insect pins and plasticene. A Grass mechanical electrical transducer was then oriented so the extensor muscles of the coxa, by moving the trochanter, would push against the extended hook of the transducer. This arrangement (Fig. 1) provided an essentially isometric situation. The transducer was connected to a Grass P-6 DC preamplifier, and the output was fed to an oscillograph or chart recorder. All nerves leaving the ganglion were severed, and two platinum electrodes were placed beneath the fifth nerve for indirect stimulation of the extensor muscles of the coxa. The four major extensor muscles in the coxa innervated by the fifth nerve are, according to the nomenclature of Carbonell (1947), muscles 178, 179D, 177E, and 179 (all these muscle units are attached to the large extensor tendon). The distal anterior surface of the coxa was removed to permit the profusion of test solutions into the muscle elements.

RESULTS

Biological Properties of Factor S

1. *Isolated ventral nerve cord*

Regions on the paper chromatograms that contained the active principle were eluted with 95% ethanol, taken to dryness, and dissolved in 1 ml. of physiological saline solution. The concentration of Factor S in the solution was then estimated

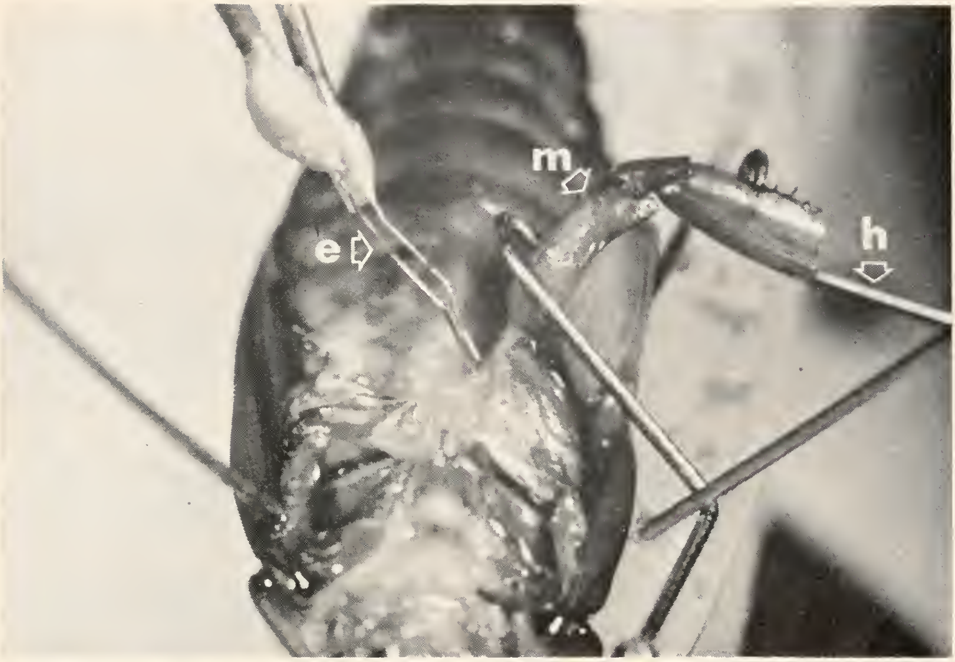

Figure 1. The cockroach nerve-muscle preparation. E = stimulating electrodes under the severed fifth nerve; H = hook from the transducer; M = exposed muscle elements.

by taking 100-μl. aliquots to dryness and weighing them on a Cahn electrobalance until a constant weight was obtained. This weight was corrected for salt content and trace contaminants from paper. In general, whole animal extracts were used as a source of Factor S. However, a number of extracts from specific tissues were evaluated. The activity of such extracts showed no difference from Factor S obtained from whole animals. In each experiment, the observation of endogenous activity on the isolated nerve cord for 10–15 minutes before applying Factor S constituted a control (Fig. 2a). Figure 2a through 2d shows a typical experiment with Factor S obtained from a whole animal extract of the American cockroach. Within 1 minute after the application of a solution containing 18 μg. of residue, the thoracic nerve cord of the cockroach showed a marked rise in arrhythmic activity (Fig. 2b). Also a two- to three-fold increase in pulse amplitude was usually observed. This excitatory response normally continued from 4 to 7 minutes and was succeeded by a rapid decline in spontaneous activity to a level considerably below normal (Fig. 2c). The cord finally became electrically silent within 5 to 10 minutes (Fig. 2d). Ordinarily, the nerve cord returned to normal levels of activity after 30 to 45 minutes in fresh saline solution. The induction of neural blockade was more rapid when the solution contained larger amounts of active residue (35 μg. or more). Also, the initial rise in arrhythmic activity was not apparent, and the blockade was more persistent. Two to three hours in fresh saline solution were required for recovery, and by that time, the nerve cord usually exhibited abnormal activity.

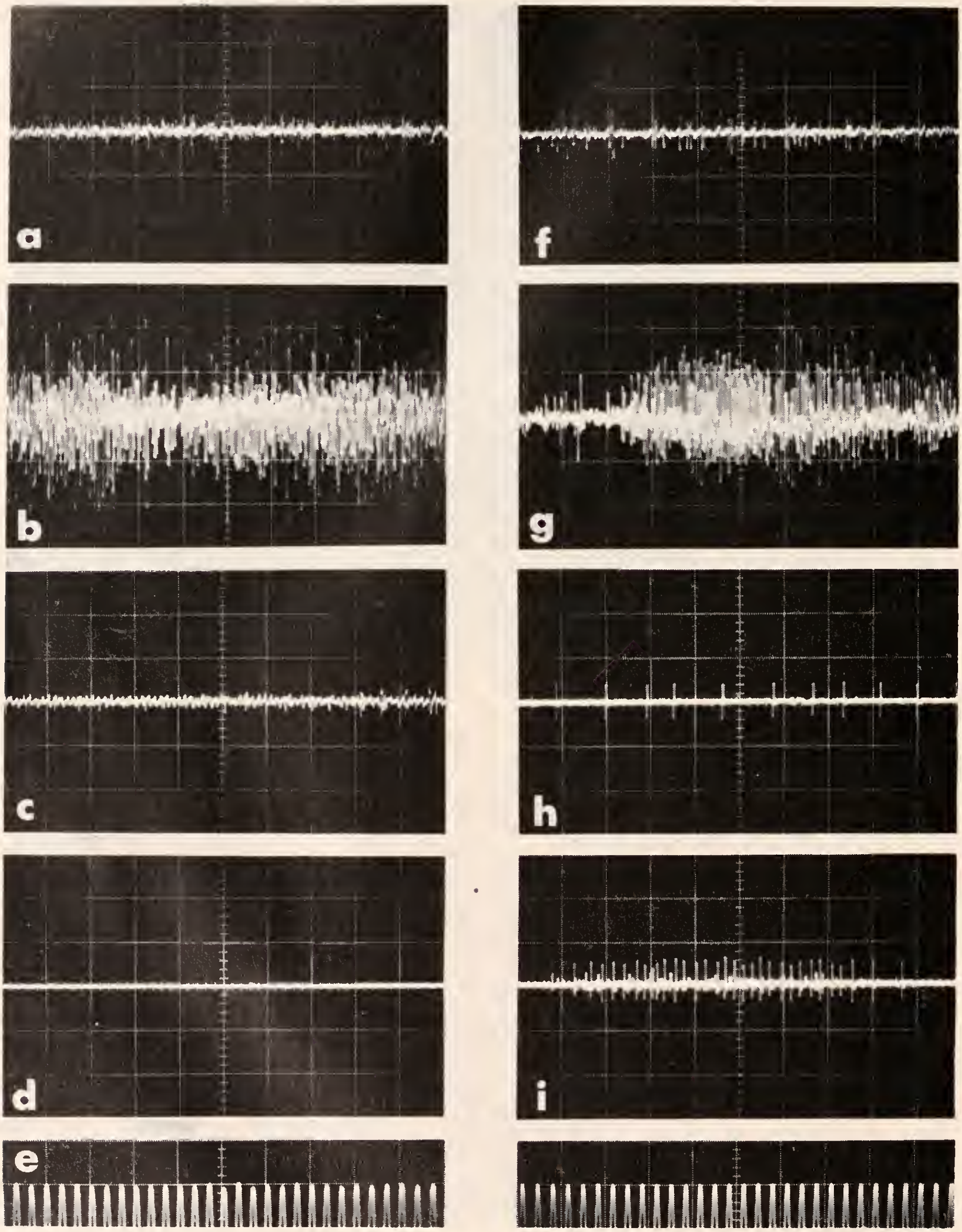

Figure 2. The response of the isolated nerve cord and motor nerve preparations to extracts containing Factor S: (a) normal activity on the isolated thoracic nerve cord of the cockroach; (b) same as (a) 30 seconds after the application of 18-μg. residue of an active sample; (c) same as (a) but 4 minutes after application of the 18-μg. residue; (d) blockade of the central nervous system 10 minutes after initial application of the 18-μg. residue sample; (e) time signals, 60 cps; (f) normal spontaneous activity of the isolated abdominal nerve cord of the crayfish in

The threshold for neural excitation with Factor S appeared to be low. The type of response recorded in Figure 2b was observed in a number of experiments with saline solution containing less than 8 μg. of residue. Active extracts obtained from the whole crayfish also excited the isolated abdominal nerve cord from the same animal (Fig. 2g). Saline solution containing 18 μg. residue induced an immediate rise in arrhythmic activity, then a depression, and finally blockade within 10 to 15 minutes.

Factor S not only increased the level of arrhythmic activity in nerve preparations, but also induced large bursts of high frequency pulses. Such bursts (Fig. 2g) were intermittent and had seemingly unpatterned recurrence. Frequently in isolated nerve cords, a large complex burst of activity occurred the instant the active sample came into contact with the preparation. Also, treated nerve cords often showed a marked facilitation after any kind of mechanical stimulation, such as that created by a drop of fresh saline solution.

The biological action of Factor S does not appear to be species-specific among arthropods. Active extracts from the American cockroach were just as effective on the isolated abdominal nerve cord of the crayfish as Factor S obtained from the crayfish itself, and vice versa.

2. *Motor-nerve preparation*

Normal activity from the motor fibers of the fifth nerve at the metathoracic ganglion is shown in Figure 2h. Figure 2i shows the response of these same nerve fibers two minutes after 15 μg. of residue was applied to the metathoracic ganglion. Impulse trains such as those shown in Figure 2i were repetitive, recurring every 2 to 3 seconds. With several preparations, muscular contractions in the mesothoracic segment occurred, and these contractions were coincident with the trains of impulses shown in Figure 2i.

3. *Nerve-muscle preparation*

Since Van der Kloot stated that Factor S had an excitatory effect on crustacean muscle, a number of experiments were made with a cockroach nerve-muscle preparation to determine what effects our active extracts might have. Figure 3 shows the potentiating effect of an active extract from the cockroach on the extensor muscles of the trochanter after a 30-second perfusion; in this instance, the concentration of Factor S is estimated at less than 20 μg. Active extracts from the house fly, *Musca domestica* (L.), produced a similar response.

4. *Occurrence and tissue distribution of Factor S*

Factor S was found in whole animal extracts of the crayfish *C. clarkii,* and the following insect species: the cockroaches *P. americana, Leucophaea maderae* (F.), and *Blaberus giganteus* (L.); the grasshopper, *Schistocerca vaga;* and the house fly, *M. domestica.* The active principle was present in the head, ventral nerve

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physiological saline; (g) same as (f) 30 seconds after exposure to a sample of Factor S (18 μg. of residue); (h) normal activity of the motor neurons in the severed 5th nerve from the metathoracic ganglion; (i) same as (h) 2 minutes after the application of 20 μg. residue of Factor S preparation to the ganglion.

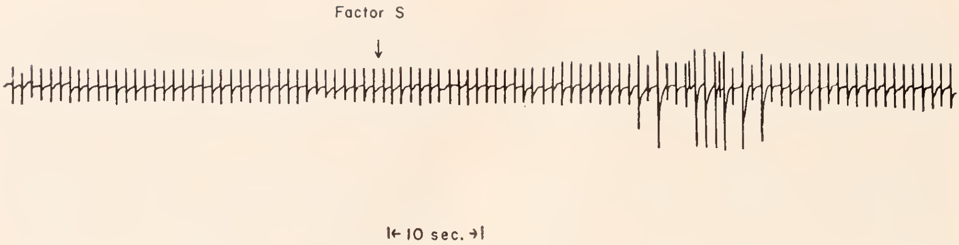

FIGURE 3. Recording of the mechanical response of the extensor muscles of the trochanter in the cockroach. Normal response to the application of a pulse strength of 6 volts every second is shown at the left. Potentiation in this response is seen at the right after a 30-second perfusion with Factor S.

cord, and the legs of the American cockroach as determined by bioassay and chemical spot tests.

The biological activity of 1-g. extracts of the ventral nerve cord were nearly equivalent to that found in 300-g. extracts of the whole animal, suggesting a high specific activity for Factor S in nervous tissue.

Chemical Properties of Factor S

1. *Extraction and purification by chromatography*

Extracts were generally prepared from 300 to 400 g. of whole insects or crayfish. Although the extraction procedure of Von Euler was normally used, several alternate methods of extraction were tried. Extraction with perchloric acid (Van der Kloot, 1960) was successful, and the active principle was also readily removed from tissues with 5% HCl in ethanol.

Initially paper chromatography was the simplest means of isolating Factor S from crude extracts. The active principle had an R_f of 0.55 to 0.65 on chromatograms developed in a butanol-acetic acid-water system and an R_f of 0.02 to 0.09 in the phenol-HCl system. Unfortunately, these chromatograms from extracts of insects and crayfish were generally streaked with a reddish-brown pigment and invariably contained a large number of fluorescent compounds. These interfering substances were removed by passing crude extracts through a 1 × 10 cm. column of IRC 50 before paper chromatography, as described previously. The initial 75 ml. of 0.2% sodium chloride contained the reddish-brown pigments and naturally fluorescent material; Factor S and such catecholamines as dopamine were eluted in the N HCl fraction. The catecholamines were readily separated from Factor S by paper chromatography in the phenol. In this system, Factor S never exceeded an R_f value of 0.1, and norepinephrine, the slowest migrating catecholamine, always had an R_f greater than 0.2 (Fig. 4).

When ethanol eluates from the phenol system were rechromatographed in butanol-acetic acid-water, the R_f of Factor S was found to be 0.15 to 0.2 (Fig. 4) in contrast to the earlier values obtained when crude extracts were simply spotted on paper. The higher R_f values were, in all probability, the consequence of cochromatography with an unknown ortho-diphenol since elutions from this R_f region often showed a major absorption band at 280, which is characteristic of

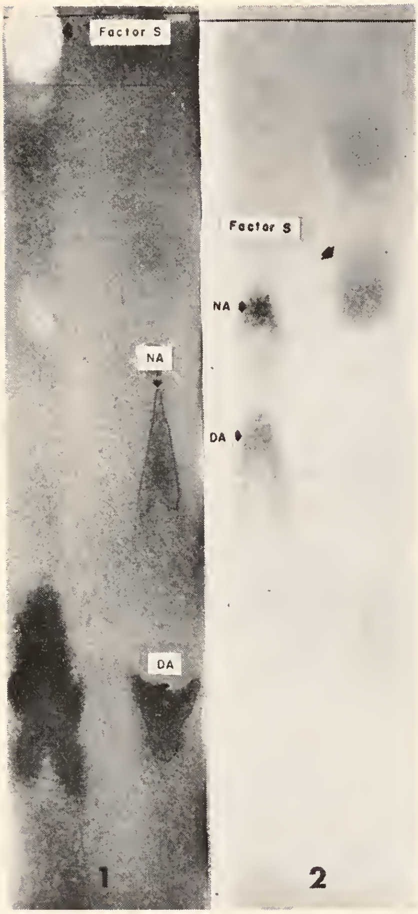

Figure 4. Paper chromatograms showing the position of Factor S; origin line is at the top of the photograph. (1): Factor S on chromatogram developed in phenol, 0.1 N HCl (1:1) and treated with potassium ferricyanide and Prussian Blue as reagents. (2): Factor S on chromatogram developed in butanol-acetic acid-water (4:1:5) and treated with 1,2-naphthoquinone-4-sulfonate. NA = norepinephrine; DA = dopamine.

many ortho-diphenols (Kamlet, 1957). This 3-phase sequence of chromatography has been used routinely to prepare samples of Factor S for chemical and biological tests. Although the purity is by no means assured, it is an improvement over previously reported isolations.

When Factor S was chromatographed in the butanol-HCl system, it again had a low R_f value (between 0.1 and 0.2) just below norepinephrine. The chromatographic behavior of Factor S in the three systems suggests a rather polar compound.

2. *Chemical reaction*

Chromatograms containing Factor S were sprayed with a number of chemical reagents. The results of these tests for specific functional groups are summarized

Table I

Functional group analysis of Factor S from the American cockroach as determined by organic spot tests

Reagent	Reaction	Functional group
Potassium ferricyanide in phosphate buffer (pH 7.7)	pink (purple UV)	a catechol or catecholamine
Ammoniacal silver nitrate	gray	a phenol, polyol, or indol
Diazotized *p*-nitroaniline	reddish-pink	a phenol or aromatic amine
2-Butanone + ammonia	negative	not a 1-substituted nicotinamide
Formaldehyde + KOH	negative	not a monoamine with a hydroxyl group in the meta position
Ethylenediamine + ammonia	negative	not an ortho-diphenol
Ninhydrin	negative	not a primary or secondary aliphatic amine
Sakaguchi test	negative	not a guanidine
Na 1,2-naphthoquinone-4-sulfonate	orange-brown	a reactive NH_2 or CH_2 group
p-Dimethylaminobenzaldehyde + conc. HCl	yellow	a primary aromatic or aliphatic amine
Diazotized sulfanilic acid	negative	not an imidazole, phenal, or aromatic amine

in Table I; the method of preparing and applying the reagents was taken either from Block *et al.* (1958) or Feigl (1960). Chromatograms sprayed with potassium ferricyanide gave a pink color in visible light and under ultraviolet light a deep blue to purple fluorescence in the region of biological activity. This reaction, for some reason, was much more consistent and intense in the phenol system than in the other developing solvents. If the chromatograms treated with potassium ferricyanide were sprayed with Prussian Blue, a bright bleached spot appeared against a blue background in the region containing Factor S (Fig. 4). The chemistry and significance of this reaction are not clear; however catecholamines generally reacted when this material was used as a reagent to give a deep blue spot against a light blue background (Fig. 4).

The potassium ferricyanide spray has perhaps had its widest use as an oxidizing agent in the detection of such catecholamines as epinephrine, norepinephrine, and dopamine (James, 1948). These amines form quite distinct and intense fluorescent products after treatment with this reagent. However, potassium ferricyanide is not specific for catechols since it can react with glucosamine and galactosamine to yield a pink color and purple fluorescence.

When areas containing Factor S were reacted with ammoniacal silver nitrate, they gave a gray color that is characteristic of phenols, polyols, and indols. The negative tests with ethylenediamine and ammonia and alkaline formaldehyde strongly suggested that Factor S is not an ortho-diphenol. Occasionally, active samples in saline solution showed a strong absorption band at 270 mμ. However, the addition of the borate ion to such a sample did not cause a bathochromic shift in the absorption. Di-ortho-substituted catecholamines generally give such a shift in the presence of the borate ion (Jurd, 1957). Norepinephrine hydrochloride, for example, when placed in insect saline solution, undergoes a 7-mμ bathochromic shift in the presence of this ion. The positive results obtained with naphthoquinone-

4-sulfonate and *p*-dimethylamino-benzaldehyde strongly suggested the presence of a primary amine.

3. *Solubility and stability*

Factor S is readily soluble in water and ethanol but not in chloroform or diethyl ether. The active principle appears to be stable in both hydrochloric and acetic acid, and it is unaffected by 10-minute exposures to an alkaline environment (pH 9). A stream of air bubbled through saline solutions containing Factor S for 50 minutes showed no detectable loss in biological activity, which suggests that it is not readily susceptible to oxidation. However, active samples in saline solution taken to dryness with an infrared lamp (75° C. for 40 minutes) lost their activity when they were resuspended. The elevated temperature does not seem to be the causative agent since active samples showed no change in the intensity of their biological response after being placed in boiling water for 10 minutes. When a 1-ml. solution of Factor S was incubated for 45 minutes at 37° C. with 35 μg. of chymotrypsin, no loss in activity was observed. Generally, preparations of Factor S retained their activity from 7 to 10 days in physiological saline solution held at 4° C. Occasionally, such samples were active for 3 to 4 weeks after storage.

Although the majority of analytical tests made to determine the chemical structure of Factor S were performed on the American cockroach, sufficient chromatographic and chemical data were obtained on the other species to permit the assumption that the material extracted from them was identical.

Discussion

Factor S, as described by Van der Kloot (1960), had an R_f of 0.6 and 0.05 in the butanol and phenol systems, respectively; it reacted with potassium ferricyanide to give a pink color and with naphthoquinone-4-sulfonate to give a yellow color. The substance we extracted from insects and crayfish had these same chemical and chromatographic properties. Van der Kloot further stated that Factor S gave a yellow color on exposure to cyanogen bromide vapor, followed by a spray of 1% *p*-aminoacetophenone in ethanol. He also observed a white fluorescence after treatment with cyanide fumes or the vapors of ammoniacal 2-butanone. On the basis of these latter tests, Van der Kloot proposed a 1-substituted nicotinamide structure for Factor S. However, the fluorescence that he observed is difficult to interpret since he made no mention of observing the natural fluorescence that is evident in extracts of both crayfish and insects. Also, ammonia alone caused either fluorescence or an increase in fluorescence in many materials that appeared in the extracts. Thus, the combination of ammonia and 2-butanone was probably not a specific test. In the cyanogen bromide p-aminoacetophenone test, we observed a number of materials in extracts which reacted with *p*-aminoacetophenone to give a yellow color, even though they had not had previous exposure to cyanogen bromide, again an indication of a lack of specificity.

In an attempt to further substantiate the 1-substituted nicotinamide hypothesis, Van der Kloot injected crayfish with 0.5 μc. of C^{14}-labeled nicotinic acid. Twenty-four hours after treatment, these animals were extracted and chromatographed on paper. When he scanned these chromatograms, he found a peak of radioactivity coincident with the R_f for Factor S. Efforts by Armson and Horridge (1964) to repeat this phase of the work were unsuccessful; they found that metabolites formed from radioactive nicotinic acid had no effect on neuromuscular transmission in the crayfish or the crab. Their evidence and the negative results we obtained with ammoniacal 2-butanone on purified samples strongly suggests that Factor S is not a 1-substituted nicotinamide.

Van der Kloot mentioned that the only substance that he could find in the literature comparable to Factor S was catechol-4 isolated by Ostlund (1954). This substance, like Factor S, was extracted by its adsorption properties on aluminum hydroxide. Ostlund found catechol-4 in several insects, the protozoan, *Noctiluca miliaris* (Suriray), the coelenterate, *Metridium dianthus* (L.), and the mollusk, *Mytilus edulis* (L.), and treatment of these chromatographed extracts with potassium ferricyanide produced a pale rose-colored spot at a low R_f in the butanol-HCl (1:1) system. However, the R_f value of this spot was not consistent with norepinephrine, epinephrine, dopa, dopamine, tyrosine, or 5-hydroxytryptamine, and the specific nature of the extraction procedure ruled out acetylcholine and histamine. When catechol-4 was chromatographed in phenol saturated with 0.1 N HCl, it had an R_f of 0.02 and 0.03 and again gave a rose color with potassium ferricyanide. Under UV light, it showed a blue color with a faint bluish-white fluorescence. The only biological property of catechol-4 observed by Ostlund was its distinct hypotensive effect upon the fowl rectal caecum. He found considerable amounts of catechol-4 in the hagfish heart, which is completely insensitive to varying doses of epinephrine, norepinephrine, dopamine, tyramine, and acetylcholine.

Our chemical results with Factor S agree with Ostlund's in almost every detail. The substance had the same R_f in butanol-HCl and phenol-HCl; it also gave a positive reaction with potassium ferricyanide and a negative one with ninhydrin.

There are three reasons for dismissing an ortho-diphenol structure for Factor S: (1) The active principle is unable to form a fluorescent condensation product with either ethylenediamine or formaldehyde. (2) No bathochromic shift in the UV spectra of active samples was observed in the presence of the borate ion. (3) The stability of Factor S in an alkaline environment is in marked contrast to the properties of most catecholamines.

Active samples were incubated with chymotrypsin since the UV spectra of the samples suggested the possibility of a peptide with aromatic amino acid residues. However, no loss in biological activity was observed after such treatment, implying that Factor S is not a peptide. This assumption is further supported by the negative tests with ninhydrin. Factor S is chemically, chromatographically, and biologically distinct from L-glutamic acid, γ-amino-butyric acid, and 5,6-dihydroxytryptamine. At the moment, the only positive information available on the chemical nature of Factor S is suggested by its solubility and the spot tests with dimethylaminobenzaldehyde and naphthoquinone-4-sulfonate. These findings indicate the presence of an amine function. The possible presence of an aromatic ring in the active principle is implied by the positive tests with diazotized *p*-nitroaniline and ammoniacal silver nitrate as well as the phenolic type absorption occasionally observed in UV.

One might speculate that Factor S potentiates neuromuscular transmission by simply increasing the number of quanta released per stimulus as 5-hydroxytryptamine does in Crustacea (Grundfest *et al.,* 1959). Prospects of such a circumstance existing, however, appear unlikely for several reasons: (1) We have observed a high specific activity of Factor S in insect neural tissue. (2) Factor S has been found in perfusates from indirectly stimulated muscles of the crayfish (Van der Kloot, 1960).

CONCLUSIONS

The substance we extracted from insects and crayfish parallels closely the biological and chemical properties of Factor S and catechol-4. Each of these substances has been designated a biogenic amine for one reason or another, and it is our contention that they are all closely related chemically, if not identical. Clearly, Factor S is implicated in the neuromuscular function of arthropods. The substance elicits an excitatory response from motor neurons in the cockroach, and it potentiates the contractions of the extensor muscles of the trochanter at low concentrations. Such features suggest that Factor S might function as a better motor transmitter in arthropods than L-glutamate; however, a more intensive physiological investigation at the cellular level is necessary to evaluate the full significance of these findings.

Since the chemical purity of Factor S is uncertain, the results of functional group and spectral analyses must be accepted with a degree of caution. Trace contamination can readily hinder organic reactions by blocking key groups in the active molecule. Results may also be obscured by the sensitivity of organic reactions. Spot tests are known to vary within wide limits, sometimes as much as 100-fold.

I am grateful for the helpful criticisms on the manuscript given by Prof. Graham Hoyle, Prof. Van der Kloot, and Dr. Morton Beroza. I also wish to express my indebtedness to Mr. George Pomonis for many stimulating and helpful discussions on the chemical aspects of this problem and to Mr. Gary R. Anderson, Mrs. Karen Soucy, Mrs. Maira de la Cuesta, and Miss Shannon Dahn for their competent technical assistance.

SUMMARY

1. A neuroactive substance has been found in extracts of the crayfish, *Cambarus clarkii* (Girard) and the following insect species: the cockroaches, *P. americana* (L.), *Leucophaea maderae* (F.), and *Blaberus giganteus* (L.), the grasshopper, *Schistocerca vaga,* and the house fly, *M. domestica* (L.). This substance in low concentrations excites motor neuron activity in the American cockroach. It also potentiates the mechanical response of the indirectly stimulated extensor muscles of the trochanter in the same insect. There is also a high specific activity of the excitatory agent in the central nervous system of the cockroach.

2. Data from extraction, chromatogaphy, and chemical analyses suggest a biogenic amine. This amine appears distinct from all commonly known neuropharmacologically active agents.

3. The chemical and biological similarities between this substance and van der Kloot's Factor S and Ostlund's catechol-4 are discussed.

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ESTERASES, PHOSPHATASES, AND GLYCOGEN IN THE ANTENNAL GLAND OF PACIFASTACUS LENIUSCULUS STIMPSON

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The excretory organs of vertebrates and certain invertebrates possess structural similarities which relate directly to their function. Extensive comparisons exist for the antennal gland of the crayfish on morphological, and to a lesser extent histochemical levels. The ultrastructural studies of Kümmel (1964), demonstrating cells in the coelomosac of the antennal gland similar to podocytes found in the vertebrate glomerulus, support the general contention that this segment of the antennal gland is the site of filtration of the primary urine (Riegel, 1963, 1965; Riegel and Kirschner, 1960). The presence of well-defined brush borders and numerous infoldings of the basal cell membranes in the labyrinth of the antennal gland (Anderson and Beams, 1956) compares to the morphology of the vertebrate convoluted tubule. The localization of alkaline phosphatase both in the brush border of the vertebrate proximal convoluted tubule (Gomori, 1941) and in the brush border of the labyrinth (Kugler and Birkner, 1948; Malaczynska-Suchcitz and Ucinska, 1962) carries the homology to the histochemical level.

This investigation was undertaken to establish the localization of the non-specific esterases, phosphatases and the distribution of glycogen in the antennal gland of the crayfish *Pacifastacus leniusculus* Stimpson. The appearance of a sexual dimorphism involving the esterases prompted the inclusion of disc electrophoresis as a means of validation and characterization.

Materials and Methods

Crayfish collected throughout the year from ponds and rivers near Corvallis, Oregon, were maintained in tanks of running cold water in the laboratory. The temperature in these tanks ranged from 10° C. in the winter to 20° C. in the summer. The size of the gastrolith and appearance of the exoskeleton were recorded to approximate the stage of molt for each animal. A total of 107 animals were used, 51 for histochemistry and 56 for gel electrophoresis.

1. *Histochemistry*

The antennal glands were fixed either in calcium-formol or in formalin-sucrose (10%–30%) for 18 hours at 4° C. The tissues were rinsed and, due to the very friable nature of the glands, were infiltrated with 15% gelatin at 37° C. for $1\frac{1}{2}$ to 2 hours. Ten-micron sections cut in the cryostat at −20° C. were mounted on

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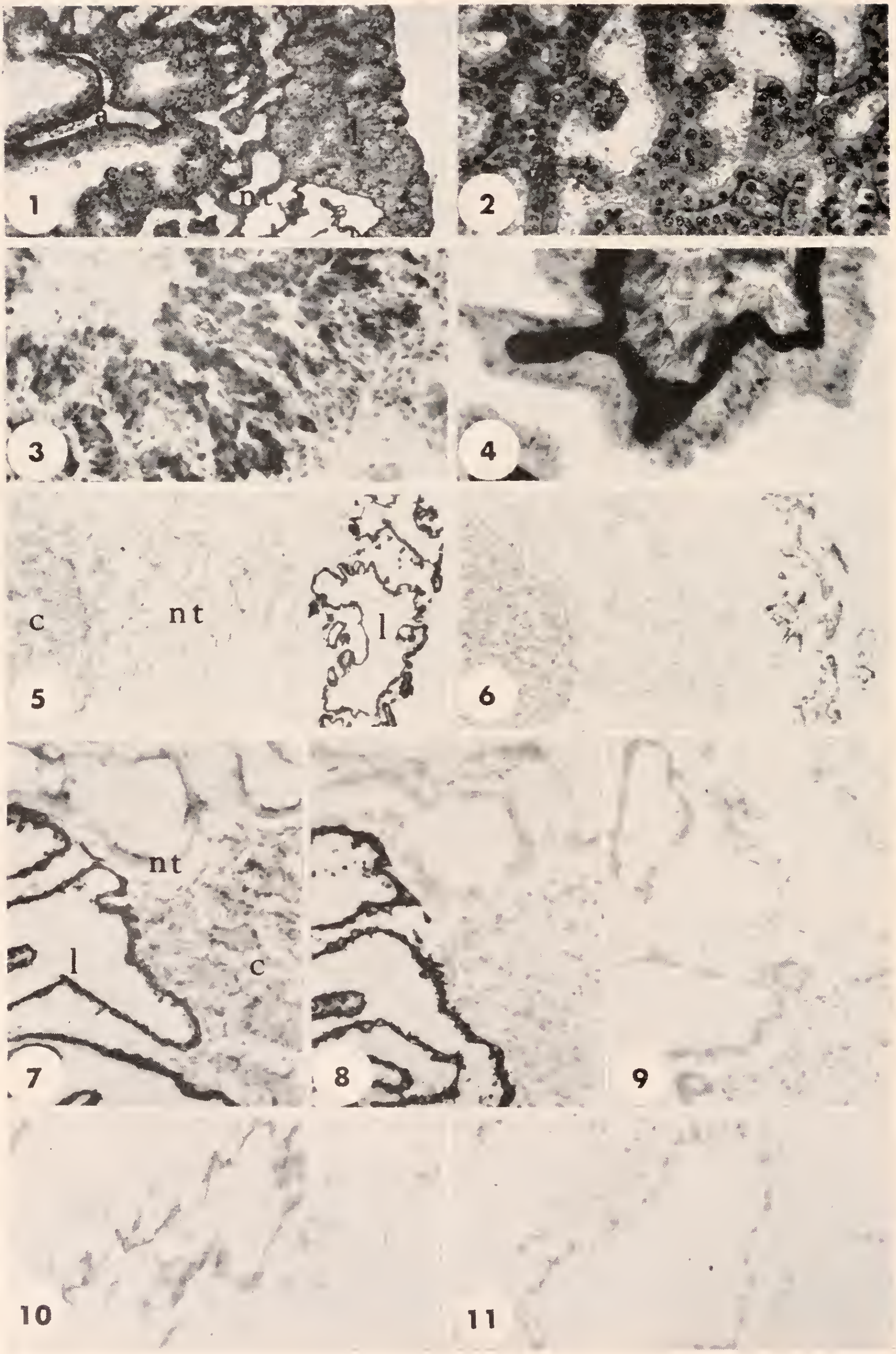
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Plate I.

cold slides and air-dried for one to two hours prior to staining. Control slides omitting substrates were processed to evaluate any non-specific staining. Alkaline phosphatase was generally demonstrated by the Gomori calcium-cobalt method but some calcium-formol-fixed tissue was also stained by the azo dye method, using fast violet B as coupler and alpha-naphthyl acid phosphate as substrate. Acid phosphatase was localized by the azo dye method, using alpha-naphthyl acid phosphate as substrate and hexazonium pararosanilin as coupler, and by the Gomori method as outlined by Barka and Anderson (1963).

The non-specific esterases were localized by the alpha-naphthyl acetate method, using hexazonium pararosanilin as coupler (Barka and Anderson, 1963). The inhibitor E 600 (diethyl-p-nitrophenylphosphate) at 10^{-5} M in 0.1 M tris maleate buffer, pH 7.2, was used to distinguish arom-esterases from the ali-esterases. Eserine sulfate at a concentration of 10^{-5} M both in distilled water and in 0.1 M phosphate buffer, pH 7.4, was used as an inhibitor of the cholinesterases (Pearse, 1961). Comparable sections were placed in either distilled water, eserine or E 600 for one hour prior to staining. Eserine was either incorporated into or omitted from the incubation medium.

Glycogen was localized by means of the periodic acid Schiff (P.A.S.) procedure as outlined by Pearse (1961). Tissues from six crayfish representing different stages of molt were fixed in Gendre fluid and embedded in paraffin. Comparable sections were subjected to a one-hour digestion in 1% diastase in distilled water prior to the P.A.S. test, to distinguish glycogen from all other P.A.S.-positive diastase-fast material.

2. *Electrophoresis*

Weighed antennal glands were homogenized in three parts of cold 0.7% saline or in distilled water and centrifuged at 12,000 *g* for 20 minutes. Normally a 100-μl. aliquot of supernatant was subjected to electrophoresis. Glands were also homogenized in 4 or 10 parts of distilled water and 100-μl., 50-μl., 25-μl., and 10-μl. aliquots of supernatant were used with no apparent differences in the final results.

FIGURE 1. Hematoxylin and eosin stained paraffin section of the antennal gland. Coelomosac (c), branch of the reno-antennal artery (a), nephron tubule (nt), and labyrinth (l). × 35.

FIGURE 2. Higher magnification of the labyrinth, demonstrating the apical vesicles both free and attached to cells. × 100.

FIGURE 3. Acid phosphatase reaction in the coelomosac stained by azo dye; formol-sucrose fixation. × 100.

FIGURE 4. Acid phosphatase reaction in the labyrinth. Stained by azo dye method; formol-sucrose fixation. × 430.

FIGURE 5. Non-specific esterase in the antennal gland of the male. Coelomosac (c), nephron tubule (nt), labyrinth (l). Formol-sucrose fixation. × 35.

FIGURE 6. Non-specific esterases in the antennal gland of the female. Formol-sucrose fixation. × 35.

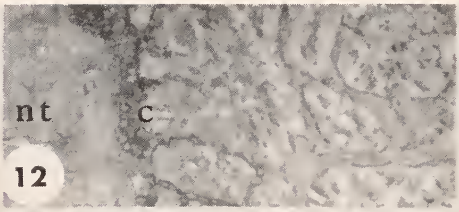
FIGURE 7. Non-specific esterases in the antennal gland of the male. Coelomosac (c), nephron tubule (nt), and labyrinth (l). Formol-sucrose fixation. × 100.

FIGURE 8. Comparable section to Figure 7 treated with 10^{-5} M eserine.

FIGURE 9. Comparable section to Figure 7 treated with 10^{-5} M E 600.

FIGURE 10. Bladder of crayfish stained for non-specific esterases. Formol-sucrose fixation. × 100.

FIGURE 11. Comparable section treated with 10^{-5} M eserine.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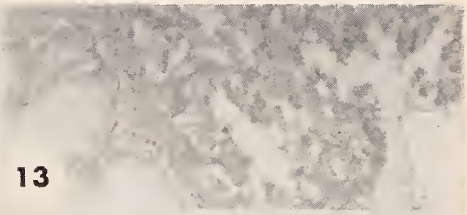
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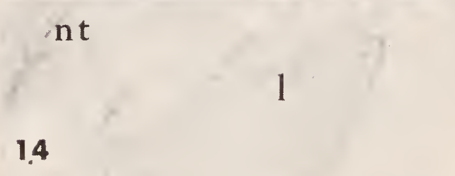
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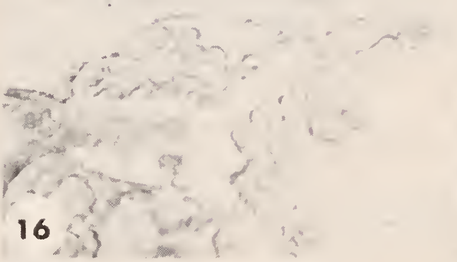
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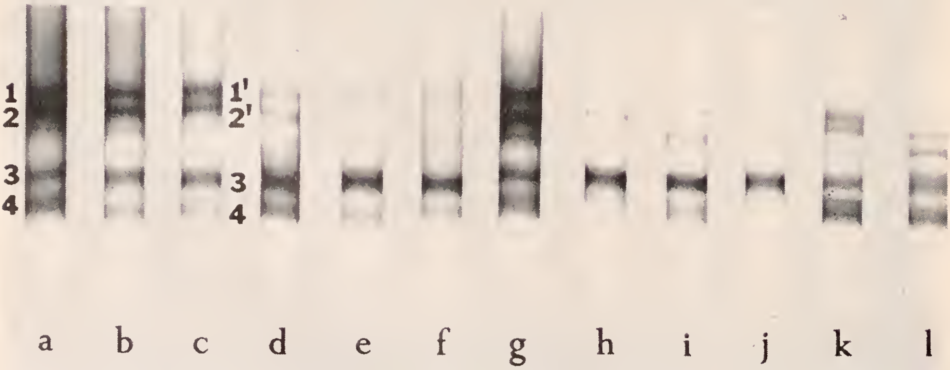
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Plate II.

In the case of extremely small antennal glands the entire supernatant constituted one sample.

The apparatus and gels were prepared according to the original recommendations of Davis (1964). The sample gel was replaced by 0.1 ml. of 40% sucrose in tris-glycine buffer containing the supernatant. This mixture formed a layer immediately above the stacking gel and was carefully overlayered with a lighter tris-glycine buffer; an initial current of 0.5 ma. was increased to 1.5 ma. after one hour, at which time the sample had migrated into the large pore stacking gel. The total time for electrophoresis was about three hours at 6° C.

Characterization of the esterases was accomplished by a one-hour exposure to cold 10^{-5} *M* or 10^{-4} *M* eserine sulfate in 0.1 *M* phosphate buffer at pH 7.4 or to 10^{-5} *M* E 600 in 0.1 *M* tris-buffer, pH 7.2, prior to the development of the gels. Control gels were soaked in cold distilled water for the same length of time. Development was accomplished in an incubation medium of the same composition used to demonstrate the esterases histochemically. Since the staining time was 30 to 45 minutes at room temperature, eserine of appropriate concentration was always included in the incubation medium of eserine-inhibited samples to prevent the reversal of the inhibitor during the prolonged incubation. Following a distilled water rinse the gels were photographed by trans-illumination to record the developed electrophoretic patterns, some of which tended to fade in storage.

RESULTS

The reader is referred to Figures 1 and 2 for an orientation to the histology of the antennal gland. More detailed descriptions of the histology appear in papers by Marchal (1892), Peters (1935), Maluf (1939, 1941), and Malaczynska-Suchcitz and Ucinska (1962).

Histochemistry

No obvious differences were observed in the histochemistry which could be correlated with the molt cycle.

1. Phosphatases

Acid phosphatase occurs as abundant granules in the cells lining the lumen of the coelomosac (Fig. 3). In the labyrinth it occupies the luminal border as an

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FIGURE 12. Section stained by P.A.S. procedure. Gendre fixation. Coelomosac (c), nephron tubule (nt). × 100.

FIGURE 13. Comparable section pretreated with diastase to remove glycogen prior to staining. × 100.

FIGURE 14. Section of labyrinth (l) and nephron tubule (nt) stained by P.A.S. Gendre fixation. × 100.

FIGURE 15. Section comparable to the above treated with diastase. × 100.

FIGURE 16. Bladder stained with P.A.S. Gendre fixation. × 100.

FIGURE 17. Bladder pretreated with diastase.

FIGURE 18. Electrophoretic patterns separated by disc electrophoresis. (a) Untreated control, adult male, (b) 10^{-5} *M* eserine, (c) 10^{-4} *M* eserine, (d) untreated control, adult female, (e) 10^{-5} *M* eserine, (f) 10^{-4} *M* eserine, (g) untreated control, adult male, (h) 10^{-5} *M* E 600, (i) untreated control, adult female, (j) 10^{-5} *M* E 600, (k) juvenile male, 45 mm. total length, (l) juvenile female, 45 mm. total length.

intense band and also appears as granules at the base of the cell around the nucleus (Fig. 4). The number and size of the granules vary to some extent between different animals. Some granules were occasionally recorded in the nephron tubule and in the bladder.

Alkaline phosphatase was present only in the labyrinth where it was typically confined to the luminal border. The nuclei presented the false positive reaction characteristic of the Gomori procedure. They were unstained when the azo dye procedure was used.

2. *Esterases*

Esterases sensitive to both eserine and E 600 are abundant in the cells lining the lumen of the coelomosac. In the labyrinth the non-specific esterases display a sexual dimorphism. The reaction in the male is very intense as compared to a much weaker reaction in the female (Figs. 5 and 6). The stain is diffuse and cytoplasmic with the apical vesicles staining intensely. Treatment with 10^{-5} M eserine does not diminish the reaction in either male or female animals but it is abolished completely by 10^{-5} M E 600 (Figs. 7, 8 and 9). The bladder and nephron tubule give a slight positive reaction which is completely eserine-sensitive (Figs. 10 and 11).

3. *Glycogen*

Glycogen is present in all areas of the antennal gland and lumen. However, cells in the periphery of the coelomosac exhibit a particularly intense diastase-labile, P.A.S.-positive stain (Figs. 12 and 13). The bladder epithelium also shows a marked staining for glycogen in comparison to the labyrinth and nephron tubule (Figs. 14, 15, 16 and 17). Additional P.A.S.-positive, diastase-fast materials are present in the nephron tubule, basement membranes, brush borders, and granules in the blood cells.

Electrophoresis

A comparison of the patterns of esterases separated from homogenates of male and female green glands supports the histochemical observations of a sexual dimorphism. In the mature male four bands are observed. The bands 1 and 2 are superimposed on a diffuse background reaction which along with band 4 is somewhat inhibited by exposure to eserine (Fig. 18 a, b, c). E 600 inhibits most of bands 1 and 2 whereas all of band 3 is not affected (Fig. 18 g, h). In the female a significant difference is observed in the bands comparable to bands 1 and 2 in the male. These bands labeled 1′ and 2′ are not as intense and do not respond to the inhibitors in the same manner as those of the male. Band 1′ is insensitive to eserine and E 600, as is its counterpart in the male, and band 2′ is sensitive to eserine (Fig. 18 d, e, f, i, j).

Crayfish of both sexes, judged to be juveniles according to the size criteria of Mason (1963), were examined to establish whether the dimorphism is present prior to their reproductive period. Homogenates of these antennal glands showed electrophoretic patterns very similar to those observed in adult animals (Fig. 18 k, l).

Discussion

The distribution of phosphatases in the antennal gland of the crayfish reported in this study confirms the presence of alkaline phosphatase at the luminal border of the labyrinth and acid phosphatase in the coelomosac as reported by Malaczynska-Suchcitz and Ucinska (1962). However, acid phosphatase was also observed as granules in the cells of the labyrinth as well as at their luminal boundaries.

The presence of both phosphatases in the same cellular area in the labyrinth raises the question as to the validity of assigning integrity to each enzyme or to placing emphasis on the possibility of a single phosphatase capable of responding to both acid and alkaline pH's. McWhinnie and Kirchenberg (1966) reported phosphatase activity in the crayfish hepatopancreas which reflected two peaks of activity: one at pH 6.8 to 7.3 and another at pH 8.0 to 8.5. In the mammalian kidney acid phosphatase and alkaline phosphatase in the proximal convoluted tubule demonstrate brush border staining which was attributed to an "alkaline" phosphatase active at an acid pH (Wachstein, Meisel and Ortiz, 1962).

Acid phosphatase-staining granules of the convoluted tubules of the rat have been accepted as lysosomes on the basis of histochemical and biochemical data (deDuve, 1963). However, the identity of the granular component in the labyrinth and coelomosac of crayfish must remain an open question until equivalent biochemical studies are performed.

The complementation of the topographical distribution of the esterases with an electrophoretic analysis of the molecular species of enzymes present is desirable and informative. However, it must be noted that the two procedures do not necessarily demonstrate the same entity. Histochemical techniques localize the insoluble enzyme fractions whereas electrophoresis demonstrates the soluble fractions (Markert and Hunter, 1959). Crayfish of both sexes exhibited E 600-resistant esterases (arom-esterases) in the electrophoretic patterns. These enzymes may represent formalin-sensitive esterases since they were not observed in sectioned material. Such an E 600-resistant, formalin-sensitive esterase has been reported in mammals (Holt, 1963).

Electrophoretic analysis revealed an E 600-sensitive esterase (ali-esterase) in the kidney of the male mouse not normally present in the females or immature males. Injections of testosterone induced its appearance in these animals (Shaw and Koen, 1963). A sexual dimorphism was also observed in the labyrinth of the crayfish which involved ali-esterases detectable by both histochemical and electrophoretic procedures.

The function of the esterases responsible for the dimorphism in mice or crayfish is unknown. For that matter, the function of the non-specific esterases in general is highly speculative. They may be either hydrolytic or synthetic and since non-specific esterases are usually isolated with the microsomes in cell fractions it has been proposed that they function in protein synthesis (Markert and Hunter, 1959; Hunter *et al.*, 1964).

Riegel (1966) proposed that the antennal gland served both in the excretion and digestion of large molecules. The "formed-bodies" observed in the urine presumably represent the structures in which these processes occur. The labyrinth exhibits secretory activity in the form of blebs or vesicles which separate from the apex of the cells to lie free in the lumen. A correlation may exist between these activities and the distribution of ali-esterases in the labyrinth of the male. Possibly the esterases associated with the dimorphism are involved in the metabolism and excretion of a substance prevalent in the physiology of male crayfish. That they are specifically localized in the labyrinth suggests a functional differentiation of this segment.

Glycogen was present in all areas of the gland, staining most intensively in the epithelium of the coelomosac and bladder and less intensely in that of the labyrinth and nephron tubule. In addition, it appeared within the lumen, thereby raising questions regarding the mechanisms of its secretion and reabsorption by the antennal gland. Possibly part of the glycogen present in the proximal areas of the gland is released into the lumen as a component of the formed bodies of the coelomosac. Since Riegel's work indicates that the formed bodies are not present in the urine of the bladder, their contents are released for reabsorption or disposal. The greater intensity of the P.A.S. reaction in the bladder epithelium may have relation to the reabsorption of glycogen.

SUMMARY

1. The esterases, acid and alkaline phosphatases, and glycogen were investigated in the antennal gland of the crayfish by histochemical procedures. Gel electrophoresis was employed to further characterize the esterases.

2. A sexual dimorphism occurred in the labyrinth characterized by an intense reaction for the ali-esterases in the male contrasted to a weak reaction in the female. Eserine-sensitive esterases were also observed in the coelomosac, nephron tubule, and bladder. Both alkaline phosphatase and acid phosphatase were localized in the luminal border of the labyrinth. Acid phosphatase was also observed in the coelomosac and as granules in cells of the labyrinth. Glycogen was most concentrated in the coelomosac and bladder but was observed in the other areas of the gland and its lumen in less concentration.

3. The significance of the above reactions was discussed in relation to their roles in the function of the antennal gland.

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DIETARY CHOLINE REQUIREMENTS FOR SPERM MOTILITY AND NORMAL MATING ACTIVITY IN DROSOPHILA MELANOGASTER[1]

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Reproduction of adult female insects is strongly influenced by the diet (Wigglesworth, 1960; House, 1961, 1962; Johansson, 1964). Nutrients required for normal female reproduction include amino acids, carbohydrate, lipid, sterol, certain minerals, and vitamins, although there is much interspecific variation. In contrast, few instances of dietary influence on male fertility have been found (Johansson, 1964). In a variety of insect species starvation results in males with smaller but functional reproductive organs than in males fed an optimal diet. Starvation also influences the sexual behavior of some species. Males of the flea *Ceratophyllus fasciatus* (Strickland, 1914) and the fruit fly *Dacus dorsalis* (Hagen, 1952) require a complete meal before they exhibit any copulatory activity. *Calliphora erythrocephala* females fed only a sugar and water diet will not accept courting males (Strangways-Dixon, 1961).

The carry-over of food stores from larval feeding poses a problem in studies of adult nutritional requirements. The small magnitude of the nutritional requirements of many adult insects, coupled with reduced feeding activity because of sizable food stores, makes depletion of carry-over food stores difficult and uncertain to obtain. Carnitine, one of the most effective substitutes for choline in the development of *Drosophila melanogaster* (Fraenkel *et al.*, 1955; Geer and Vovis, 1965), was used in the present study to obtain choline-free *D. melanogaster* adults. No larvae are able to pupate on a diet not supplemented with choline or a related compound. When carnitine is fed at a concentration equivalent to the optimal choline level in the larval diet, nearly as many larvae develop to eclosion as when choline is fed, but the larval growth period is 20% greater in duration (Geer and Vovis, 1965). Carnitine-raised adults are morphologically normal but they contain no detectable choline in their tissues. When carnitine-raised males and females are mated, however, they fail to reproduce (Geer, Vovis and Yund, 1967).

The current study presents evidence for a dietary choline requirement for the development of motile spermatozoa in *D. melanogaster* and quantitatively defines the requirement. Choline-deficiency is also shown to influence the normal sexual behavior of adult *D. melanogaster*.

METHODS AND MATERIALS

Adults for test purposes were derived from the Canton-S, Riverside and Oregon-R strains by a three-way mating scheme. Canton-S females were crossed

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[1] This investigation was supported by National Science Foundation Grant GB-4838.

to Riverside males and the hybrid female offspring were in turn mated to Oregon-R males. Eggs collected from females of the latter mating were sterilized by rinsing them thoroughly with sterile distilled water, washing with a 0.125% sodium hypochlorite (commercial Clorox) solution to eliminate egg-clumping, and exposing the eggs to a 0.4% peracetic acid-0.1% sodium alkylarylsulfonate solution for 10 minutes. Eggs were rinsed in 70% ethanol before transfer to cultures.

A defined medium consisting of amino acids, sucrose, yeast RNA, cholesterol, B vitamins and salts prepared as an agar gel as described by Geer and Vovis (1965) was employed for the main part. However, adults for mating activity observations and determination of male and female responses to dietary choline were raised on a diet containing 3.5% casein in place of the amino acid mixture; the modification of Sang's medium C (Sang, 1956) reported by Geer (1963). The choline requirement for reproduction was influenced in no way by this alteration of the dietary nitrogen source. Unless otherwise indicated, the diets were supplemented with either 5.7×10^{-4} *M* DL-carnitine HCl or $5.7 \times 10^{-4} M$ choline chloride. Cultures were maintained at 23.8° C. in 6-dram shell vials containing 5 ml. of medium. These were sterilized by autoclaving for 20 minutes at 15 lb./in.^{2} pressure.

Males and females to be crossed to mates fed different test diets were separated within 12 hours after eclosion, using sterile laboratory instruments to avoid contamination of cultures. Cultures found to be contaminated during the course of the experiments were discarded. Males and females crossed to mates fed the same diet were moved to fresh cultures soon after eclosion. Egg production and the hatchability of eggs laid by females were determined by transferring the females with their mates to cultures for a 24-hour period, removing the adults, and then assessing the number of eggs laid and number to hatch within 24 hours after being laid. Observations of mating behavior were conducted by placing male and female pairs into 6-dram vials without anesthetizing and recording the time from introduction to copulation. Bastock and Manning's (1955) description of *D. melanogaster* courtship served as a guide for observations. The spermathecae and seminal receptacles of females that had copulated were dissected out in Drosophila Ringer's solution (Ephrussi and Beadle, 1936) and examined for sperm to determine whether the females had been successfully inseminated by their mates.

The development of motile sperm was ascertained by dissecting out the testes of adult males in Drosophila Ringer's solution and examining mature sperm for motility. The morphology of living spermatozoa was studied by phase microscopy or spermatozoa were stained with aceto-orcein or aniline blue-eosin (Gurr, 1965) before examination by light microscope methods.

In this paper the hatchability of eggs laid by females mated to test males is used as an assay for the development of motile sperm in the males. Results presented in the next section show that egg hatchability lags only slightly behind the appearance of motile sperm in the testes of males. Thus, the assay for motile sperm depends upon the frequency of hatchable eggs laid by females to increase as the quantity of motile spermatozoa increases in the testes of their mates.

The sources of chemicals were as follows: low vitamin casein—Gentosan Division, Fisons Pharmaceuticals Limited, Loughborough, Leicestershire, England; other nutrients and vitamins—Nutritional Biochemicals Corporation, Cleveland,

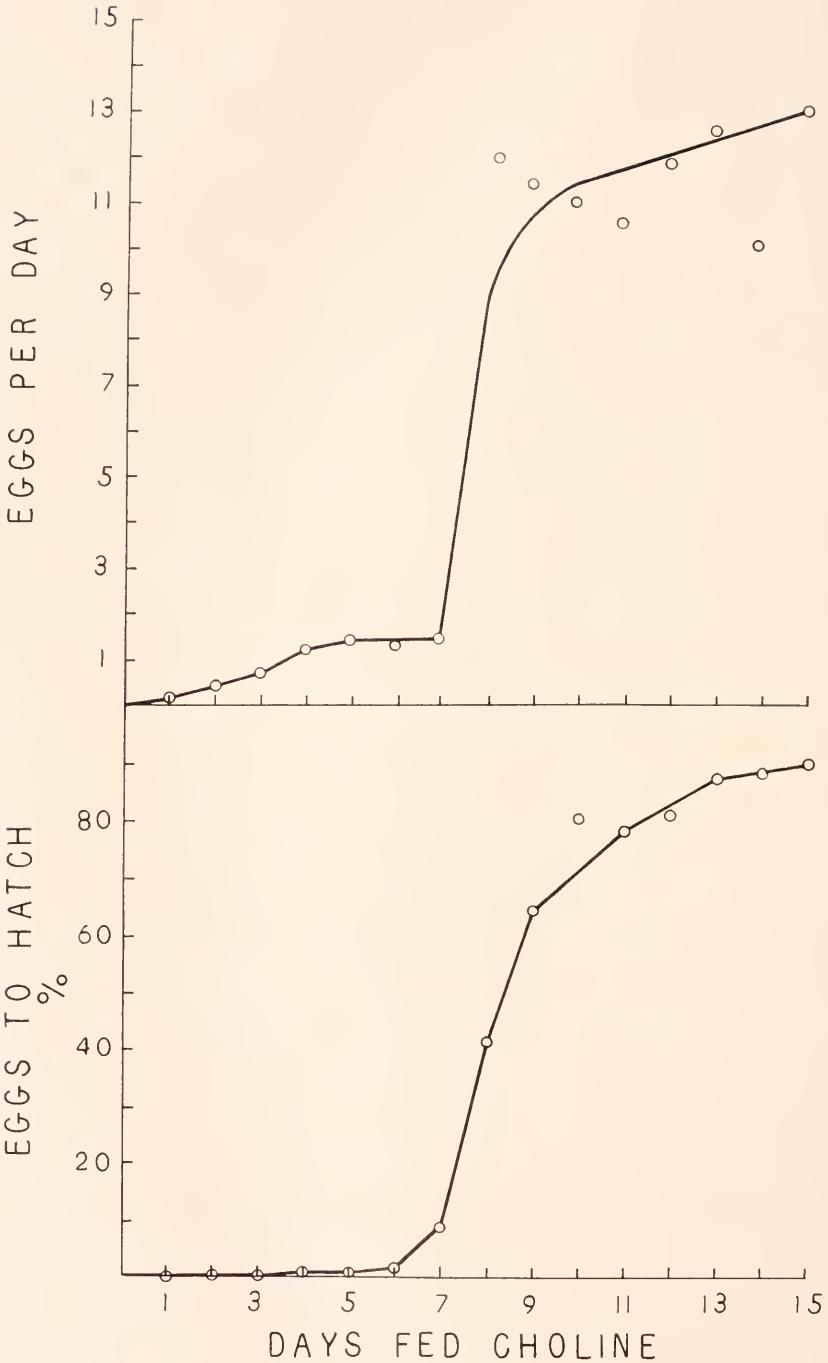
EGGS PER DAY
15
13
11
9
7
5
3
1
% EGGS TO HATCH
80
60
40
20
1
3
5
7
9
11
13
15
DAYS FED CHOLINE

Figure 1.

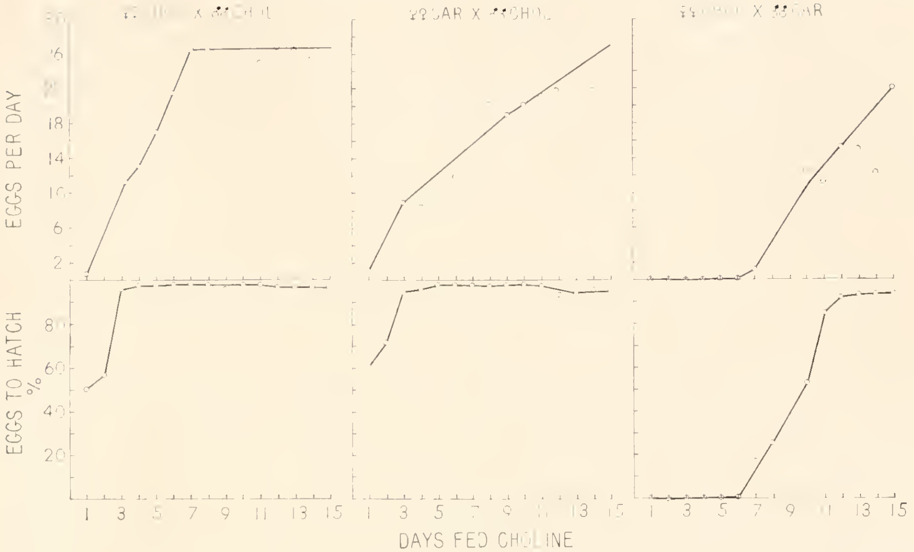

Figure 2. The reproductive capacities of choline-raised females mated to choline-raised males (left), carnitine-raised females mated to choline-raised males (center), and carnitine-raised females mated to carnitine-raised males (right) when both mates were fed a diet containing 5.7×10^{-4} *M* choline. The upper half of the graph indicates the eggs laid per female per day and the lower half shows the percentage of eggs to hatch for each day of the test period. Circles in the left graph represent mean values for 40 females and 50 males in 5 cultures, circles in the center graph are for 120 females mated to 120 males in 12 cultures, and circles in the right graph are for 96 females mated to 120 males in 12 cultures.

Ohio; inorganic salts—J. T. Baker Chemical Company, Phillipsburg, New Jersey; 40% peracetic acid—Inorganic Chemicals Division, FMC Corporation, New York, New York; sodium alkylarylsulfonate—Fisher Scientific Company, Chicago, Illinois; Orcein—Eastman Organic Chemicals, Rochester, New York; aniline blue—National Aniline and Chemical Company, Inc., New York, New York; Eosin B—Matheson Coleman Bell Division, Matheson Company, Inc., East Rutherford, New Jersey.

Results

Response to dietary choline

The initial investigation was to find if the sterility of carnitine-raised adults could be corrected by feeding choline. Adults raised on a carnitine-supplemented diet were fed a diet containing 5.7×10^{-4} *M* choline for 15 days. The number of eggs laid per female and the percentage of these eggs to hatch was determined for each day during the test period (Fig. 1). Almost none of the eggs hatched

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Figure 1. The reproductive capacity of carnitine-raised females mated to carnitine-raised males when both mates were fed a diet containing 5.7×10^{-4} *M* choline. The upper half of the graph indicates the eggs laid per female per day and the lower half shows the percentage of eggs to hatch for each day of the test period. Circles represent mean values for 80 females mated to 100 males in 10 cultures.

TABLE I

Increase of sperm motility in carnitine-raised males fed a choline-containing diet

Days fed choline	Number of males	Males with motile sperm
0	12	0
4	12	0
5	12	2
6	12	3
7	12	8
8	12	12

until the seventh day, then the hatchability increased steadily for the next 9 days. Females raised on a carnitine diet also laid very few eggs. After choline was added to the diet, the egg productivity of females remained low until adults had been fed choline 7 days; then egg productivity jumped from slightly more than one egg per female per day to more than 10 eggs per female per day.

The responses of carnitine-raised males and females to dietary choline were compared by crossing carnitine-raised males and females to choline-raised mates and determining the egg hatchabilities and egg production of the females of these two matings when maintained on a choline diet (Fig. 2). Choline-raised males and females were mated as a control. Carnitine-raised females when mated to choline-raised males laid a relatively large quantity of eggs after 2 days on a choline diet and 71.5% of the eggs hatched. Egg hatchability for carnitine-raised females increased rapidly, reaching an optimal level at the end of day 5 of the choline feeding period. These results were similar to those of the choline control matings. Egg production continued to climb throughout the test period but did not reach the productivity level of the choline control matings until after 15 days of feeding.

Carnitine-raised males, on the other hand, did not reproduce until after 7 days of choline feeding. Eggs laid by their choline-raised mates began to hatch after day 7 and egg hatchability increased through day 15, reaching a level only slightly less than that of the choline control matings. Egg production increase paralleled the climb in egg hatchability but did not attain the levels reached by choline- or carnitine-raised females when mated to choline-raised males.

TABLE II

The reproduction of D. melanogaster grown on a carnitine-supplemented diet and fed either a choline- or carnitine-supplemented diet for 8 days as adults

Mating*	Total eggs	Eggs to hatch (%)	Egg productivity (eggs/female/day)
♀♀ ♂♂			
Carnitine × Carnitine	31	0	1.3
Carnitine × Choline	170	11.1	7.7
Choline × Carnitine	73	0	4.3
Choline × Choline	309	74.4	14.7

* Adults were mated for 4 days while being fed a carnitine-supplemented diet before being tested.

TABLE III

The reproduction of D. melanogaster grown on either a choline- or carnitine-supplemented diet and maintained on a carnitine-supplemented diet for 7 days as adults

Mating	Total eggs	Eggs to hatch (%)	Egg productivity (eggs/female/day)
♀♀ ♂♂			
Carnitine × Carnitine	14	0	0.4
Carnitine × Choline	295	39.6	9.8
Choline × Carnitine	41	0	1.7
Choline × Choline	201	84.5	7.7

The choline response experiments show that the sterility of carnitine-raised adults is primarily due to the inability of males to reproduce, a condition that can be corrected by feeding choline. Upon examination, the testes of carnitine-raised males were found to be devoid of motile sperm although spermatozoa in all stages of development were present. When choline was fed, some males possessed motile sperm after 5 days of feeding but all males did not have motile sperm until after 8 days of feeding (Table I). The development of motile sperm preceded only slightly the increase in hatchability of eggs laid by their mates.

Choline fed during the larval period

Matings were made between carnitine-raised adults maintained on a carnitine diet and those fed a choline diet (Table II). Carnitine-raised and -fed adult females when mated to carnitine-raised and choline-fed males laid relatively large numbers of eggs, and a significant percentage of the eggs hatched, 11.1%. Carnitine-raised and fed males were sterile since females mated to these males laid very few eggs and none of the eggs hatched.

Choline obtained by feeding during the larval period was sufficient to insure the fertility of adult males (Table III). Results similar to those described for the previous matings were obtained when adults raised on either choline- or carnitine-supplemented diets were mated. Fewer of the eggs laid by carnitine-raised females

TABLE IV

The effect on male fecundity of adding choline to the diets of larvae fed a carnitine-supplemented diet

Larva age when choline added* (days)	Larvae to become adults (%)	Growth period† (days)	Eggs to hatch‡ (%)
3	81.3	12.0 ± 1.1	96.6
6	80.0	12.0 ± 1.1	95.5
8	76.6	12.4 ± 1.0	88.8
10	69.6	13.3 ± 1.0	95.6

* Eight mg. of choline chloride were added to each culture in two drops of 70% ethanol.

† Figures represent mean ± S.D. The growth period was the time from inoculation of the cultures with eggs until the larvae pupated

‡ The adults were fed a carnitine-supplemented diet 6 days before being tested.

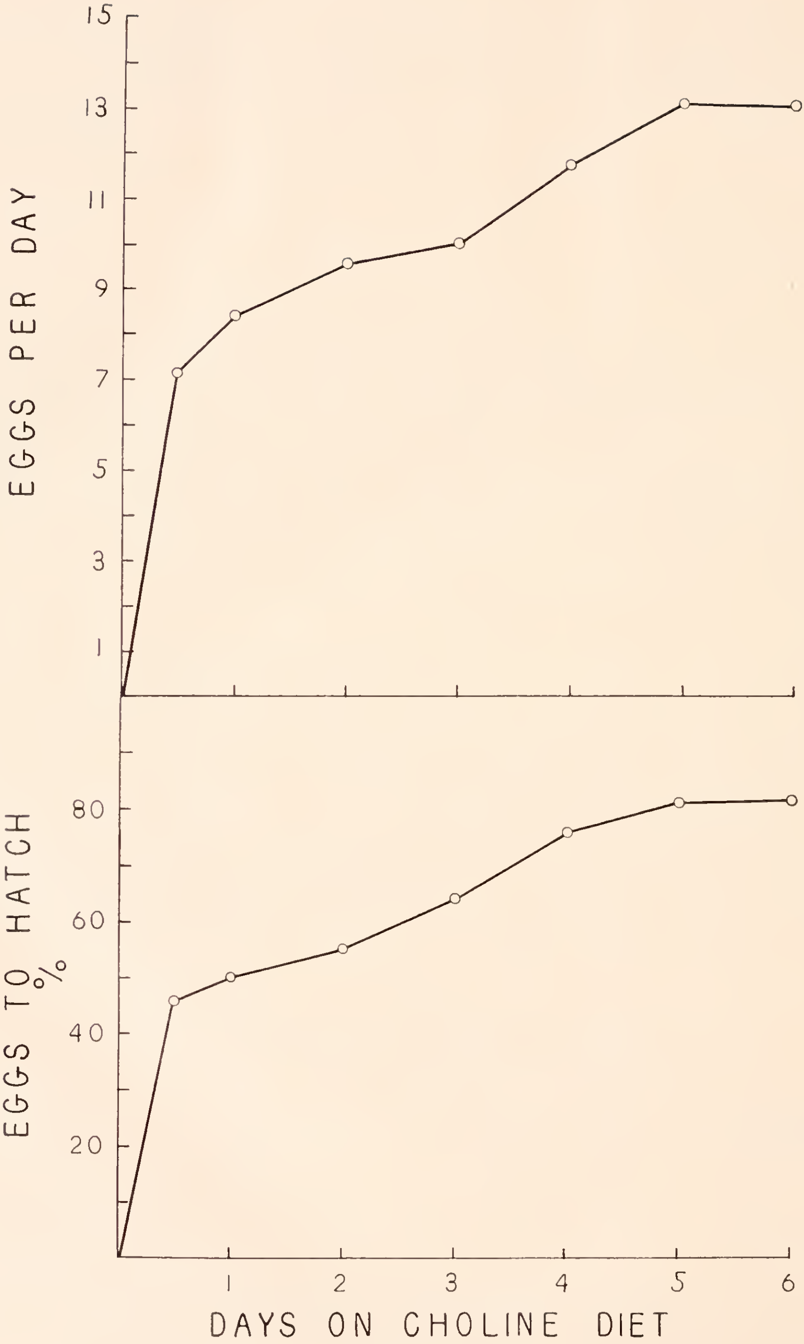
EGGS PER DAY
15
13
11
9
7
5
3
1
EGGS TO HATCH %
80
60
40
20
1
2
3
4
5
6
DAYS ON CHOLINE DIET

FIGURE 3.

TABLE V

The mating activity of males and females raised and maintained as adults for 7 days on a diet supplemented with either choline or carnitine

Female diet	Male diet	Number of pairs observed	Pairs to copulate within $2\frac{1}{2}$ hours	Average time to copulation (seconds)	Number of females inseminated
Choline	Choline	20	14	2133 (437–3327)*	14
Choline	Carnitine	20	5	3058 (1219–4991)	0
Carnitine	Choline	20	3	6164 (2293–8565)	3
Carnitine	Carnitine	20	1	6741	0

* The range in time to copulation of mating pairs is indicated in parentheses.

mated to choline-raised males hatched than did when choline-raised females were mated to choline-raised males. Carnitine-raised males were sterile regardless of the larval diet of their mate.

Choline fed late in the larval period appears sufficient to insure adult male fertility (Table IV). Although there was a significant extension of the larval growth period and a decrease in the number of larvae to become adults, the percentage of hatchable eggs mated to the eclosing males remained at a high level. It is possible that a more curtailed larval choline feeding period might result in a measureable degree of sterility; nevertheless, enough choline is consumed during a 3-day larval meal for sperm motility.

Mating behavior

Observation of the mating activity of carnitine- and choline-raised adults showed that mating was inhibited by choline deprivation (Table V). Carnitine-raised and -fed males copulated more readily with choline-raised and -fed females than carnitine-raised and -fed females, but could not successfully inseminate females. Carnitine-raised and -fed females, though they did not copulate readily, were successfully inseminated by choline-raised and -fed males when copulation occurred. Carnitine-raised females appear less receptive to male courtship advances than do their choline-raised counterparts. Carnitine-raised and -fed males court vigorously but seldom get beyond the licking stage of courtship with carnitine-raised and -fed females (the stage preceding mounting and copulation). When they do, the males have difficulty mounting the females. That male activity is also inhibited by choline deprivation is shown by the rapid and successful mating of choline-raised and -fed males when mated to choline-raised and -fed females as compared to carnitine-raised and -fed males when mated to choline-raised and -fed females.

FIGURE 3. The reproductive capacities of carnitine-raised adults maintained on diets supplemented with 5.7×10^{-4} M choline for periods ranging from $\frac{1}{2}$ to 6 days. All test groups were aged to 8 days on carnitine-supplemented medium before being tested. The upper half of the graph indicates the eggs laid per female per day and the lower half shows the percentages of eggs to hatch for each test group. Circles represent mean values for 80–120 males and females in 6 cultures for each test group.

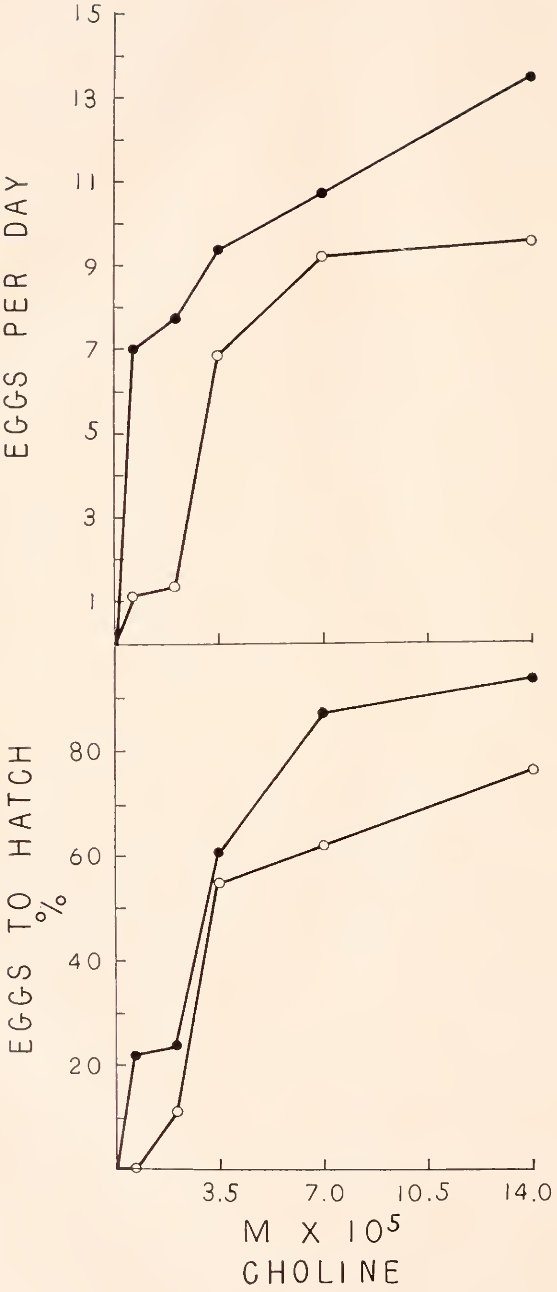
EGGS PER DAY
15
13
11
9
7
5
3
1
% EGGS TO HATCH
80
60
40
20
3.5
7.0
10.5
14.0
M X 10^5
CHOLINE

FIGURE 4.

Choline feeding period

Because a lag period of several days occurred before carnitine-raised males became fertile, experiments were performed to find if a long choline adult feeding period is necessary for the development of motile sperm or whether a short choline feeding period plus a period on a choline-free diet are sufficient. Carnitine-raised adults were fed a diet containing 5.7×10^{-4} *M* choline for periods of time ranging from 0 to 6 days and maintained on a carnitine-supplemented diet until 8 days old. As little as ½ day on a choline diet increased egg hatchability significantly and subsequent feeding up to 5 days on a choline diet further increased egg hatchability (Fig. 3). Egg hatchability for the females fed a ½-day choline meal was 46.2%, increasing to 81% for females fed choline for 5 days. Egg production followed a similar pattern. Egg production for females fed choline for ½ day was 7.1 eggs per female per day, whereas females given a 5-day meal laid an average of 12.9 eggs per female during the test day. Thus, if choline is fed at a concentration of 5.7×10^{-4} *M*, 5 days of feeding as an adult are needed to accumulate sufficient choline for optimal male fertility.

Concentration of dietary choline

Concentration of choline in the diet, as well as the length of the choline feeding period, was found to be important in the degree of fertility attained by carnitine-raised adults. To assess the effect of dietary choline-concentration, carnitine-raised adults were fed diets containing from 0.7 to 14×10^{-5} *M* choline and then were fed the diets for 4 more days and retested. Distinct differences existed between the hatchabilities of these eggs laid by females fed the test diets for 8 days (Fig. 4). None of the 45 eggs hatched that were laid by females fed a diet with 0.7×10^{-5} *M* choline but 10.3% and 55.1% of the eggs hatched laid by females fed 2.1 and 3.5×10^{-5} *M* choline, respectively. Egg productivity was slightly more than 1 egg per day for females fed a diet containing either 0.7 or 2.1×10^{-5} *M* choline, but was 6.9 eggs per day for females fed 3.5×10^{-5} *M* choline. Both egg productivities and egg hatchabilities for adults fed 7 and 14×10^{-5} *M* choline were also markedly improved by the higher choline levels.

Egg productivity and egg hatchability were higher for adults fed choline for 12 days instead of 8 days regardless of the concentration of choline. Egg hatchabilities had risen to 22.3% and 23.4%, and egg productivity had risen to 7.0 and 7.7 eggs per female for the two lower choline levels tested. There was a continuous gradient from 9.3 to 13.5 eggs per female per day and 61.5% to 92.0% hatchability for the higher choline levels fed. Clearly, choline can be accumulated in the tissues of the adults, the rate of accumulation depending upon the dietary choline concentration and the amount accumulated depending upon the duration of the feeding period. These results show that the development of motile sperm is dependent upon the accumulation of a minimal amount of choline but degrees of

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FIGURE 4. The reproductive capacities of carnitine-raised adults maintained on diets supplemented with 0.7 to 14×10^{-5} *M* choline for 8 days (open circles) and 12 days (filled in circles). The upper half of the graph indicates the eggs laid per female per day and the lower half shows the percentages of eggs to hatch for each test group. Circles represent mean values for 72 to 110 males and females in 6 cultures for each test group.

fertility between absolute sterility and optimal fertility result when limiting amounts of choline are fed.

Choline substitutes

Betaine, 2-dimethylaminoethanol and homocholine were fed to larvae with carnitine to see if these combinations would stimulate sperm motility (Table VI). Larval growth was influenced but not adult fertility. Betaine was without effect whereas 2-dimethylaminoethanol and homocholine shortened the larval growth period. 2-Dimethylaminoethanol also reduced the number of larvae to become adults. Adults raised on diets supplemented with carnitine and either 2-dimethylaminoethanol or homocholine may have produced a slightly greater quantity of eggs than adults fed only carnitine. These eggs, however, failed to hatch, indicating the absence of motile sperm.

Adults raised on a diet supplemented with either homocholine or β-methylcholine alone were also found to be sterile. Homocholine-raised adults produced more eggs than carnitine-raised adults, whereas β-methylcholine adults produced no more eggs than carnitine-raised adults. Thus, homocholine appears to stimulate Drosophila females to oviposit when they have not been inseminated with motile sperm.

The abilities of choline-related compounds to promote male fertility were also tested by feeding carnitine-raised adult diets supplemented with one of the related compounds. The fertility of these adults was tested after 8 and 12 days of feeding. Table VII summarizes the present findings and reviews the activities of the test compounds in promoting larval growth and development. None of the nine choline-related compounds was effective in promoting male fertility. In contrast, all but betaine have some activity in promoting adult growth and development. Thus, the requirement for sperm motility is quite specific for the intact choline molecule, much more specific than the requirement for development.

TABLE VI

The effects on adult reproduction of feeding larvae choline-related compounds

Larva supplement*	Larvae to become adults (%)	Growth period† (days)	Eggs to hatch‡ (%)	Egg productivity (eggs/female/day)
Carnitine	73.5	14.1 ± 1.5	0	0.2
Carnitine + betaine	72.5	14.5 ± 1.8	0	0.2
Carnitine + 2-dimethylaminoethanol	61.1	13.1 ± 1.1	0	0.7
Carnitine + homocholine	70.3	12.1 ± 1.1	0.9	1.3
Homocholine	71.0	12.2 ± 1.1	0	1.9
β-methylcholine	72.8	13.1 ± 1.6	0	0.2

* Carnitine was fed in equimolar amounts with other supplements. The total supplement concentration was 5.7×10^{-4} *M* in all diets.

† Figures represent mean ± S.D.

‡ The adults were fed a carnitine-supplemented diet 8 days before being tested.

TABLE VII

Comparison of the activities of choline-related compounds in the development and fertility of Drosophila melanogaster

Compound	Development†	Male fertility
Choline	++*	++
Monoethylcholine	++	−
Diethylcholine	+	−
2-Dimethylaminoethanol	++	−
2-Methylaminoethanol	+	−
Carnitine	++	−
β-methylcholine	++	−
Homocholine	++	−
Sulfocholine	+	−
Betaine	−	−

† Taken from Geer and Vovis (1965).

* ++ indicates at least 60% as effective as choline, + less than 60% as effective as choline, and − ineffective. All compounds were tested at 5.7 × 10^{-4} *M*.

Sperm morphology

Spermatozoa of carnitine-raised males have been examined with the light microscope. There is no indication of morphological abnormalities but, as in Kiefer's study (1966) of the spermatozoa of X/O males, examination with the electron microscope may reveal structural deficiencies. The numbers of mature sperm in terms of sperm bundles were reduced in carnitine-raised males.

DISCUSSION

To interpret the results of the present study, the position of carnitine-raised Drosophila in terms of choline metabolism must be clarified. During the growth period of the first generation cultured on a carnitine-supplemented diet, larvae are able to use carnitine as a choline substitute effectively. If provided with adequate quantities of carnitine, nearly as many larvae pupate and become adults as when choline is fed, although the growth period is slightly extended (Geer and Vovis, 1965). When carnitine supplementation is delayed, larvae that have been maintained on choline-free diets become increasingly less able to utilize carnitine as well as choline (Geer, Vovis and Yund, 1967). Carnitine is less than 10% as effective as choline in promoting development during the pupal period and, as indicated in the present study, the adult male is incapable of using carnitine as a choline substitute for sperm motility. Bridges, Ricketts and Cox (1965) have also noted that adult *Musca domestica* cannot readily incorporate carnitine into their tissues.

Carnitine-raised *D. melanogaster* adults contain no measureable phospholipid-bound choline in their tissues (Geer, Vovis and Yund, 1967); yet, a phospholipid is present that is very similar chromatographically to the lecithin of the choline-raised adults (Geer and Dates, unpublished). The lecithin-like compound is probably similar to the β-methylcholine-containing phospholipid isolated from carnitine-raised *Phormia regina* (Bieber *et al.,* 1961; Bieber, Cheldelin and Newburgh, 1963).

Carnitine may act by replacing choline in certain metabolic activities, thus releasing choline for those activities for which carnitine is an ineffective substitute. Experiments being conducted in this laboratory indicate that significant amounts of choline are derived by larvae from both the egg and sperm. Delay in supplying dietary carnitine may result in the depletion of sperm- and egg-transmitted choline, thus the reduced effectiveness of carnitine as a choline substitute for the offspring of choline-fed adults when supplementation is delayed. An alternate explanation is that the physiological activities for which choline is required are less specific during early development than during the pupal period or adult stage.

Choline-related compounds are known to supplement the activity of choline in development when fed with choline in the diet. Choline is several times more effective when fed in suboptimal quantities if 2-dimethylaminoethanol is also included in the diet (Geer, Vovis and Yund, 1967). 2-Dimethylaminoethanol when fed alone sponsors the development of only a small percentage of larvae to the adult stage. Carnitine, however, does not complement the activity of 2-dimethylaminoethanol, indicating further the existence of choline-specific developmental activities. Carnitine-raised adults are morphologically normal but possess no detectable choline in their tissues for physiological activities that specifically require choline. One can speculate that the ability to utilize choline-related compounds such as carnitine for certain metabolic activities is of adaptive value to dipterous insects since these insects lack the ability to synthesize choline. Carnitine is widely distributed in nature and might be available under some circumstances when choline is the limiting dietary factor.

The choline requirement of carnitine-raised adult males for sperm motility is very specific. None of the choline substitutes exhibiting activity in development are active in sponsoring sperm motility. This is consistent with the interpretation of both specific and less specific activities for choline in insect metabolism.

Although the primary reason for the sterility of carnitine-raised adults is the immotility of spermatozoa, a secondary reason is the ineptness of both carnitine-raised females and males in courtship. Carnitine-raised males are more successful in courtship with a choline-raised mate than are carnitine-raised females when crossed to a choline-raised mate. Also, carnitine-raised males copulate more readily with choline-raised females than with carnitine-raised females. Several observations of courtship activity indicate that carnitine-raised males court females vigorously but seldom get beyond the licking and probing stage. When they do, they have difficulty in mounting the female. Carnitine-raised females are less active than their choline-raised counterparts and are less receptive to the advances of the male. However, the differences in mating activities of carnitine- and choline-raised adults are quantitative since carnitine-raised males and females have been observed to copulate but carnitine-raised males never successfully inseminate the female, due to the absence of motile sperm.

Carnitine-raised females do not require choline for oogenesis since they lay large numbers of viable eggs after mating with choline-related males. This agrees with the observation by Sang and King (1961) that choline-raised females do not have a choline requirement for normal oogenesis.

Three variables must be determined to adequately define the choline requirement for sperm motility. There is a distinct lag period after choline is fed before adult males become fertile. Some variation exists in the time that individual males become fertile but all males possess motile sperm by the eighth day on a choline-supplemented diet. No attempt was made to quantify the number of motile sperm after choline feeding periods of different lengths but differences were evident. It seems likely that choline is utilized in the formation of motile sperm rather than activation of the mature immotile sperm since the lag period is great enough for this process.

Although there is a lag period of five to eight days before motile sperm are formed if choline is fed at a dietary level of 5.7×10^{-4} M, choline may be accumulated in the body if present in a lower concentration in the diet so that sperm motility may ensue though delayed. If choline is supplied at a dietary concentration of 1.4×10^{-4} M, for example, 12 days of feeding is necessary for optimal male fertility.

The length of the feeding period for optimal male fertility for a choline level of 5.7×10^{-4} M was 5 days if a sufficient lag period was allowed before testing. In fact, choline-feeding periods as short as ½ day were effective in promoting male fertility with a 7½-day time lapse before testing. Thus, to define the choline requirement for the development of motile sperm in *D. melanogaster* males, the choline concentration in the diet, the length of the feeding period, and the time period between the initial choline meal and fertility test must be stated. A time lapse sufficient for uptake of a minimal amount of choline into the adult body, incorporation of choline into the reproductive tract, and successful insemination of females with motile sperm are necessary. Thus, the choline requirement for optimal fertility of *D. melanogaster* males may be stated as a feeding period of 5 days on a diet supplemented at a level of 5.7×10^{-4} M with a total time lapse of 12 days between the initial feeding and examination of eggs laid by females mated to the test males. Eight days are required for the appearance of motile sperm in the testes of all males after choline is fed, whereas the choline response experiments indicate that reproduction is not at an optimal level until 12 days after the initial choline meal. The number of motile sperm must reach an optimal level and mating must occur between day 8 and day 12.

The existence of a dietary choline requirement for spermatozoan motility in Drosophila is significant in light of observations on sperm metabolism in other animals. The primary lipids of spermatozoa are known to be choline-containing phospholipids, the principal phospholipid of invertebrate spermatozoa being lecithin (Mohri, 1957; Hartree and Mann, 1959; Barnes and Dawson, 1966) with choline plasmalogen and lecithin both being prominent in vertebrate spermatozoa (Lovern *et al.*, 1957; Hartree and Mann, 1959; Gray, 1960; Scott, Dawson and Rowlands, 1963; Hartree, 1964; Bratanov, Dikov and Angelova, 1965; Minassian and Terner, 1965).

Phospholipid serves as an endogenous energy source for spermatozoan motility. Lardy and Phillips (1941a, 1941b) first suggested this after observing that bull spermatozoa washed free of seminal plasma maintain motility under aerobic conditions. Under anaerobic conditions in the presence of the carbohydrate of seminal plasma, energy for the motility of many vertebrate spermatozoa is supplied by fructolysis preferentially (Mann, 1946, 1954; Gonse, 1962). Under aerobic sugar-free conditions some mammalian spermatozoa metabolize choline plasmalogen selectively in lieu of lecithin, the fatty acids of choline plasmalogen being utilized as a substrate in spermatozoan respiration (Carlson and Wadstrom, 1958; Hartree and Mann, 1959, 1961; Hartree, 1964). Phospholipid may also serve as the source of energy for mammalian spermatozoa during the maturation period in the epididymis (Scott, Voglmayr and Setchell, 1967). In contrast to the capacity of mammalian spermatozoa to acquire energy by aerobic or anaerobic means, invertebrate spermatozoa, which are shed into an aquatic environment without the benefit of an accessory fluid, are dependent upon the oxidation of intracellular phospholipid as an energy source (Rothschild and Cleland, 1952; Mohri, 1957, 1964; Gonse, 1962).

Whether Drosophila spermatozoa resemble either vertebrate or marine invertebrate spermatozoa in the utilization of phospholipid as an energy source is unknown. Certain observations suggest the importance of phospholipid in the metabolism of Drosophila spermatozoa and also indicate resemblances to the vertebrate pattern. Faludi, Csukás-Szatlóczky and Széplaky (1960) found that dietary P^{32} is incorporated into the lipid of Drosophila during the larval and pupal developmental periods and that adult males raised on a P^{32}-containing diet contribute an amount of P^{32}-containing lipid to their offspring sufficiently large to influence the larval lipid composition. Other reports indicate that the primary source of phospholipid in the male ejaculate is the seminal fluid and not the spermatozoa and that the fluid is critical to male fertility. King (1954) observed that dietary phosphorus accumulated during a 24-hour adult feeding period, a period too short for the incorporation of phosphorus into sperm DNA, is stored in the accessory glands and ejaculatory bulb and is released from these glands with ejaculated sperm during copulation. Oftedal (1959) confirmed that the most important pathway of phosphorus transfer from male to female during the mating of *D. melanogaster* is the seminal fluid, and Lefevre and Jonsson (1962) found that inability to transfer sperm due to excessive mating results not from lack of mature sperm, but from the lack of accessory gland secretion.

The synthetic capacities of vertebrate spermatozoa are well documented. Human, fish and bull spermatozoa can synthesize phospholipid readily (Terner and Korsh, 1962; Minassian and Terner, 1966) using glucose or glycerol as substrates for the glycerol moiety and acetate as substrate for fatty acids. Scott, Dawson and Rowlands (1963) noted an increase in choline plasmalogen content of rat spermatozoa as they passed through the epididymis and Terner (1965) found that human spermatozoa can use glucosamine of the cervical secretion as substrate for energy metabolism and synthesis of lipids. Utilization of maternal derivatives by Drosophila spermatozoa has been suggested but, as yet, not adequately demonstrated. Anderson (1945), following studies of the *lozenge* mutant of *D. melanogaster* which lacks or has defective spermathecae, suggested that substances essential to the survival of sperm in the seminal receptacles of the female are derived from the spermathecae, a questionable hypothesis since Bender and Green (1962) have shown that the low reproductive capacities of *lozenge* females may largely be due to ovarian pathologies. Herskowitz (1963) demonstrated a maternal effect on restitutional events leading to the healing of breaks induced in mature sperm chromosomes. The rate of paternal mutations increased 50% and the rate of partial loss of the paternal sex chromosomes increased 300 to 600% in eggs oviposited by females undernourished before mating and irradiation, but did not increase greatly when females were well fed. Although the maternal effect shown by Herskowitz may not be representative of normal sperm metabolism, the utilization of nutrients derived from the female by spermatozoa seems almost a certainty due to the relatively long period of sperm storage in Drosophila females. Although vertebrate and Drosophila spermatozoa may share the capacity to use exogenous nutrients derived from tissues of the female reproductive tract, the permeability of spermatozoa of marine invertebrates such as *Spisula* and *Arbacia* to exogenous substrates is known to be limited (Gonse, 1962; Hartree, 1964).

The requirement for choline for Drosophila spermatozoa motility is dictated by two factors: (1) the inability of Drosophila to synthesize choline from available substrate substances (Geer, Vovis and Yund, 1967); and (2) the requirement for choline as a constituent of phospholipid. Utilization of choline-containing phospholipid as an energy-yielding substrate has not been demonstrated but is strongly suggested by the present study. Another need for choline for normal sperm function is suggested by the postulate that acetylcholine esterase activity is critical to coordination and propagation of the flagellar wave of spermatozoa (Tibbs, 1962; Nelson, 1964). The present experimental results are also consistent with Nonidez's observation (1920) that sperm motility is critical for the successful insemination of female Drosophila.

The current study represents the first case of sperm immotility in Drosophila associated with a dietary requirement. *D. melanogaster* males that lack the Y chromosome are sterile and the production of functional sperm is dependent on seven fertility factors located in the Y chromosome (Brosseau, 1960). By employing electron microscopy methods, Kiefer (1966) found abnormal development of Nebenkern derivatives and incomplete axial fiber complexes in the spermatozoa of X/O males. In another case of male sterility in *D. melanogaster,* Shoup (1967) found that a translocation of parts of the X chromosome to chromosome 2 blocks differentiation of the sperm head as well as preventing the formation of arginine-rich histone.

Gene activity in the Y chromosome critical for the development of functional sperm in Drosophila has been observed. Chromosomal differentiations of the lampbrush type in the Y chromosomes of *D. hydei* and *D. neohydei* in growing spermatocytes are necessary for the formation of spermatozoa (Hess and Meyer, 1963). Meyer, Hess and Beerman (1961) found crystalline structures suggestive of a metabolic block and noted that nuclear structures were missing in the spermatocytes of males lacking a Y chromosome.

Genes necessary for the utilization of choline in the development of spermatozoa may be located in the Y chromosome. It is possible that some of the seven heterochromatic genes essential for the fertility of *D. melanogaster* males (Brosseau, 1960) may be active in the utilization of dietary choline. This, of course, is only speculation, but it is a possibility that warrants investigation.

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SUMMARY

1. Adult *D. melanogaster* raised on a carnitine-supplemented diet fail to reproduce unless choline is included in their diet. The sterility is due primarily to a lack of motile sperm but carnitine-raised adults also mate much less readily than choline-raised adults. Carnitine-raised females are fertile, however, when inseminated by choline-raised males. Supplementation of the diet with 5.7×10^{-4} *M* choline for 5 days will correct the sterility of carnitine-raised males provided 7 additional days elapse before the fertility test. All males possess some motile sperm by day 8 following the initial choline meal but females mated to test males do not lay eggs that hatch at the optimal level until day 12. Thus, 5 days of feeding are required for the accumulation of sufficient choline for optimal fertility but it is not until 7 days after the choline feeding period that a maximum number of motile sperm are formed. A choline meal of less than 5 days in duration results in less than optimal male fertility, whereas a feeding period longer than 5 days is required for optimal fertility if choline is fed at a concentration less than 5.7×10^{-4} *M*. The choline requirement for the development of motile sperm is very specific; betaine, homocholine, sulfocholine, diethylcholine, monoethylcholine, carnitine, β-methylcholine, 2-dimethylaminoethanol, and 2-methylaminoethanol failed to substitute for choline.

2. Choline may be required for the synthesis of phospholipid needed as an energy source for sperm motility. This requirement would be similar to the requirements of many vertebrate and invertebrate spermatozoa for a choline-containing phospholipid as an endogenous energy source for motility. Since *D. melanogaster* can not synthesize choline, the choline needed for sperm motility must be supplied by the diet.

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ENDOCRINOLOGY, REGENERATION AND MATURATION IN NEREIS

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The processes of growth and reproduction make heavy demands on the resources of an organism. It is not surprising to find that, as Clark (1962) pointed out, there is an incompatibility between growth and reproduction, though the degree of separation of the two processes varies considerably. In insects reproduction is delayed until molting and growth are completed. Herlant-Meewis (1962) remarked that the processes of scissiparity, involving somatic growth, and sexual reproduction do not occur together in the Naididae. According to Watson (1962), there is a different type of separation in Thysanura. Ecdyses and growth continue in the reproductive adult but reproduction and growth alternate during each instar. Limb regeneration occurs in the juvenile but not in the adult cockroach (Bodenstein, 1955; 1959) nor in adult decapods because of the essential nature of the molting hormone (Passano and Jyssum, 1963).

Among syllid polychaetes, specialized growth processes associated with reproduction lead to the development of stolons which bear the gametes. However, in more typical polychaetes, the growth rate declines as maturation proceeds (Clark, 1962; Clark and Clark, 1962; Clark and Scully, 1964).

Hormonal mechanisms effecting a separation between growth and reproduction have been demonstrated in nereids, and as in many other animals, the secretion of a juvenile hormone is involved. The supraesophageal ganglion, or brain, of nereids secretes a hormone or hormones which promote segment growth but inhibit maturation during the early stages of development (see reviews by Durchon, 1962; Clark, 1965; and Hauenschild, 1966). Immature nereids regenerate lost posterior segments. This segment growth is dependent on the presence of the brain hormone both for its initiation and its further progress (Casanova, 1955; Durchon and Marcel, 1962; Clark and Ruston, 1963b; Hofmann, 1966; Golding, 1967a, 1967b). However, Durchon (1965) has reported that 4-month-old *Perinereis cultrifera,* having about 50 segments, is able to regenerate several times over after the removal of the prostomium. Unpublished observations by the author indicate that very young *Nereis limnicola,* having about 20 segments, is unable to regenerate posterior segments in the absence of the prostomium. Possibly *Perinereis* differs from *Nereis* in this respect.

Several authors have noted the inability of mature *Nereis diversicolor* to regenerate segments (Stephan-Dubois, 1956; Clark and Ruston, 1963b; Scully, 1964). Clark and Ruston (1963b) and Scully (1964) investigated this phenomenon by designing experiments to test the following two possible explanations: (a) that mature animals do not regenerate because of a failure on the part of the supraesophageal ganglion to secrete the necessary hormone, or (b) that they do not regenerate because their tissues have become incapable of responding to the hormone.

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Both investigations yielded results which were interpreted to mean that the first of these two explanations is the correct one.

The experiments described below were designed to reinvestigate the effect of maturation on regeneration and to shed light on the endocrine mechanisms involved in the control of these processes.

Materials and Methods

N. diversicolor was collected from the littoral zone of the River Avon, Bristol, England, and maintained in the laboratory in 50% sea water. Extirpation of the supraesophageal ganglion was carried out by the removal of the intact organ, together with the overlying epidermis. All experiments involved determination of the maximum oöcyte diameter of each of the animals used. This was accomplished by placing the animal on dry filter paper and puncturing the dorsal body wall about half-way along the length of the body with a fine glass capillary tube. The tip of the latter was ground on carborundum paper in order that it might cause as little damage as possible. Coelomic contents were forced into the tube by the internal hydrostatic pressure. A drop was blown out onto a coverglass which was inverted over a cavity slide. The maximum oöcyte diameter was measured by microscopic observation and the use of a calibrated micrometer eye-piece.

Further details, including those describing grafting techniques, have been given elsewhere (Golding, 1967a, 1967b).

Statistical significance of data was determined by the use of the *t*-test.

Results

Regenerative ability and maturity

In the first experiment, the regenerative ability of animals at various stages of maturity was investigated. Six groups of animals were used, each group consisting of 10 specimens, and each being at a different stage of maturity. The first group was made up of animals in which oöcytes (or spermatocytes) had yet to be shed into the coelom. The other groups consisted of animals whose maximum oöcyte diameters were 21–60 μ, 61–100 μ, 101–140 μ, 140–180 μ, and above 180 μ, respectively. The number of segments possessed by each animal was determined and the mean

Table I

Regenerative ability and maturity

Maximum oöcyte diameter (μ)	0	20–60	61–100	101–140	141–180	180+
Mean no. of segments, initially	67	72	79	80	83	90
No. of animals	10	10	10	10	10	10
No. surviving 21 days	10	8	10	9	9	9
Mean no. of regenerated segments	11.0	11.9	10.5	8.0	2.1	0
S. E.	0.8	0.5	0.9	1.5	0.8	—

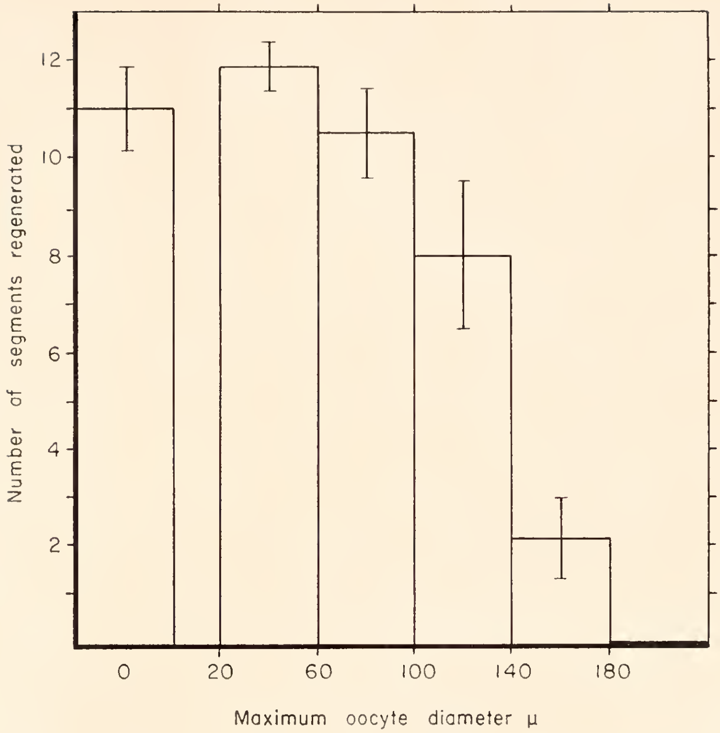

FIGURE 1. The regenerative ability of *Nereis diversicolor* at different stages of maturity.

number calculated for each group. Supraesophageal ganglia were allowed to remain *in situ*. Forty segments were amputated from each animal. After 21 days, the number of segments regenerated by each specimen was determined. The results are expressed in Table I and Figure 1.

One difficulty with respect to this experiment was that the amputation of 40 segments from each animal involved the removal of a greater proportion of the body of immature worms than that amputated from more mature ones, since the latter possess a greater number of segments. However, for the sake of simplicity, this factor was ignored.

It is clear that the regenerative capacity does not vary significantly during the stages of development represented by the different groups, until the maximum oöcyte diameter exceeds 140 μ. The mean number of segments regenerated by the group containing oöcytes 101–140 μ in diameter is less than that regenerated by the group with oöcytes 61–100 μ in diameter, but the difference is not statistically significant. However, the group of animals with oöcytes of 141–180 μ in diameter regenerated significantly fewer segments than less mature groups ($P < 0.01$), but significantly more than the most mature group, which regenerated no segments ($P < 0.05$).

Experiments were designed to determine whether the regenerative impotence of mature worms is due to an inability on the part of the supraesophageal ganglion

TABLE II

The secretory activity of the mature ganglion

Age of donors Age of hosts	Immature Immature	Mature Immature	Mature Mature*
No. of animals	20	20	30
No. surviving 21 days	19	18	20
Mean no.	9.5	4.3	0.1
S. E.	0.7	0.6	—

* Mature animals, with *in situ* ganglia; 17 out of 20 regenerated a pygidium.

to secrete regeneration hormone or to an inability of the body to respond to the hormone.

The secretory activity of the mature ganglion

In this experiment, 30 mature animals, each containing oöcytes $> 180\,\mu$ in diameter, were first tested for regenerative ability. This was accomplished by amputating 40 segments from each. The ganglion was left *in situ*, since it has been shown previously (Golding, 1967a), that in animals capable of regenerating, it invariably induces more regeneration in this situation (in other words, the most stringent test for regenerative ability was applied to these animals). Another reason for leaving the ganglia *in situ* was that implanted ganglia are difficult to retrieve from the coeloms of mature animals, due to the opacity of the body wall. The jaws were removed and the animals kept together in aerated water for 21 days, after which they were examined.

As Table II (third column) shows, these animals were almost completely incapable of regeneration. Only 2 specimens regenerated one pair of parapodial rudiments each.

Forty immature animals (each with a maximum oöcyte diameter less than $120\,\mu$), each 65–75 segments long, were divided into 2 groups of equal size. Forty segments and the ganglion were removed from each member of one group. The ganglia of the mature animals which had been tested and found to be incapable of regeneration were then transplanted into these decerebrate, tail-less hosts. The other group of immature animals acted as a control. Forty segments were removed from each, and each ganglion was transplanted into the coelom of another member of the group (so that no animal received its own ganglion as an implant). Both groups of hosts were kept together in one bowl of aerated water. They were separable into their respective groups due to distinctive parapodial clipping, carried out as described previously (Golding, 1967a). After 21 days, the number of segments regenerated was determined. The results are given in Table II.

The results show that ganglia removed from mature animals, incapable of regenerating, induce the proliferation of a significant number of segments in immature, tail-less hosts. However, they are not as effective as the ganglia of younger worms ($P < 0.01$).

In the second experiment, 50 immature animals, containing oöcytes $< 120\,\mu$ in diameter, and having 65–75 segments, were divided into five groups of equal size.

Forty mature animals, containing oöcytes > 200 μ in diameter were divided into four groups of equal size. The supraesophageal ganglion and all but 35 anterior segments were removed from each of the first group of immature *Nereis*. These animals received implants of the ganglia of one of the groups of mature animals. The implanted ganglia were located and removed from the coelom after five days and replaced by ganglia freshly extirpated from mature donors. This procedure was carried out every five days so that each of these hosts had finally been subjected to the influence of four ganglia removed from mature animals, consecutively, each ganglion remaining in the coelom for five days after its initial extirpation.

The second group of immature hosts were prepared in the way described for the first group. They received implants of the ganglia implanted into, and removed from, that group. Thus each member of the second group was subjected to the influence of four ganglia, implanted and removed one after another. Each ganglion remained in the coelom of the host from the 6th to the 10th day after initial extirpation.

The ganglia were implanted into the third group of hosts after removal from the second group. In this way each member of the third group was subjected to the influence of four ganglia, consecutively, each ganglion remaining in the coelom from the 11th to the 15th day after its implantation into an immature host.

By transplantation of the ganglia from the third to the fourth group, the latter were subjected to their influence from the 16th to the 20th day after their extirpation.

The fifth group constituted a decerebrate control, all but 35 segments being removed as for the other groups. Mock operations were carried out every five days.

The results are given in Table III. The numbers of segments regenerated by the four groups of hosts into which ganglia were implanted do not differ significantly. The results show that ganglia of mature animals secrete regeneration hormone during the first five days after their implantation into immature hosts, and that the subsequent rate of secretion is no higher than that during this time.

The competence of the mature host

This aspect of the problem was investigated by an experiment involving transplantation of ganglia from immature donors into mature hosts.

The regenerative ability of 20 immature animals (the maximum oöcyte diameter not exceeding 120 μ), each 65–75 segments long, was tested by implanting each ganglion into the coelom of another member of the group, and the removal of 40

TABLE III

Transplantation of mature ganglia

Time in days after extirpation	1–5	6–10	11–15	16–20	Control
No. of animals	10	10	10	10	10
No. surviving	10	10	10	10	9
Segments regenerated	4.1	4.3	3.9	3.1	0.6
S. E.	0.4	0.4	0.6	0.6	0.3

TABLE IV

The competence of the mature host

Age of donors	Immature	Immature	Mature
Age of hosts	Immature	Mature	Mature
No. of hosts	20	19	20
No. surviving 21 days	19	14	15
Mean no.	8.5	0	0
S. E.	0.5	—	—

segments from each. They were kept together for 21 days, after which the number of segments regenerated was determined (Table IV, first column). These animals were thus shown to be capable of prolific regeneration of segments, and their ganglia to be very active, endocrinologically speaking.

Forty mature animals, each containing oöcytes at least 180 μ in diameter, were divided into 2 groups of equal size. The first had 40 segments and the ganglion removed from each member, and received implants of the ganglia of the young animals, each ganglion being retrieved from the coelom of its immature host.

Forty segments were also removed from each member of the other group of mature animals. The ganglion of each was transplanted into the coelom of another member of the group.

Both groups of mature hosts were kept together, in one bowl of aerated water, for 21 days.

The results are given in Table IV. They show that neither the control group nor hosts with ganglia from immature donors regenerated segments. A mature worm cannot be made to regenerate by the implantation of an immature ganglion.

Neurosecretion in the mature animal

Although ganglia excised from mature animals, which have lost the ability to regenerate, induce a significant amount of regeneration when implanted into immature, decerebrate, tail-less hosts, this does not necessarily mean that secretion of hormone occurs *in the mature animal.*

TABLE V

Neurosecretion in the mature animal

	Hosts	Grafts
No. of animals	30	30
No. surviving 21 days	36	26
No. showing no regeneration	25	11
No. regenerating 1 segment	0	7
2 segments	1	7
3 segments	0	1
Mean no.	0.1	0.9
S. E.	—	0.2

TABLE VI

Effects of transplanting immature ganglia into grafted Nereis

	Immature ganglia implanted		No ganglia implanted	
	Host	Graft	Host	Graft
Number of animals	10	10	10	10
No. surviving	10	10	9	9
Segments regenerated	2.3	2.4	0.8	1.3
S. E.	0.4	0.4	0.4	0.4

In this experiment, fragments of worm prepared from immature animals were grafted into mature hosts. The grafts originated from animals 65–75 segments long, containing oöcytes not more than 120 μ in diameter. The hosts contained oöcytes at least 180 μ in diameter. Forty posterior segments were amputated from each host, and at least 40 from each graft. After 21 days, the numbers of segments regenerated by hosts and grafts were determined.

The results are expressed in Table V. They show that the hosts were virtually incapable of segment regeneration (one specimen regenerated 2 pairs of parapodial rudiments) which would be expected in view of their maturity. The extent of regeneration in the grafts was rather less than that which occurs in decerebrate animals at a comparable stage of development (for comparison, see Golding, 1967a, Table 2).

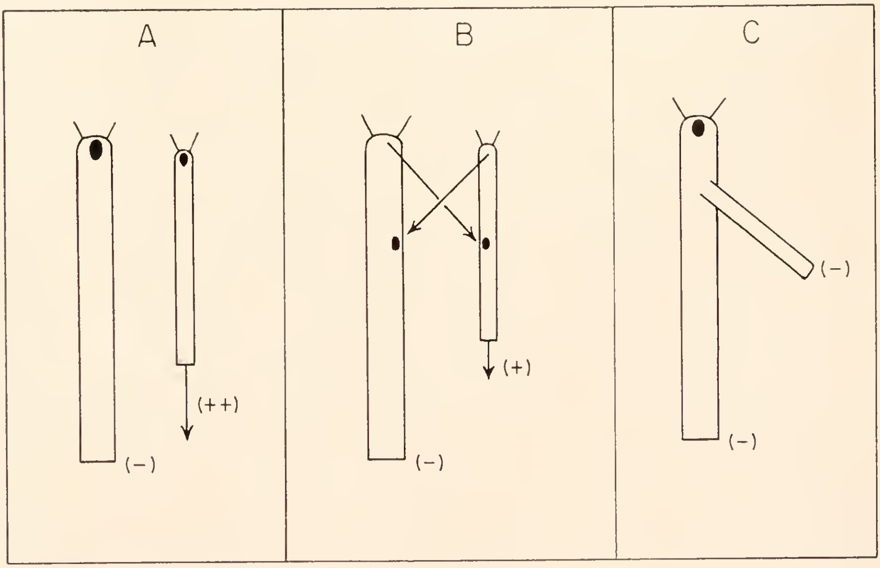

FIGURE 2. Diagrammatic representation of the principal experiments and their results. See text for detailed explanation.

In the second grafting experiment, 10 grafts were stitched into 10 hosts. Each graft consisted of segments 13–32 of an immature *Nereis,* containing oöcytes $< 120\,\mu$ in diameter, and having originally 60–75 segments. Each host was mature, containing oöcytes $> 200\,\mu$ in diameter. Forty segments were removed from each. Three ganglia originating from immature donors were implanted into each graft.

To provide a control, 10 comparable grafts were stitched into 10 mature hosts. Immature ganglia were not, however, implanted.

The two groups of grafted animals were kept for 21 days, after which the number of segments regenerated by hosts and grafts was recorded. The results are given in Table VI. They show that implantation of a number of immature ganglia induces a significant ($P < 0.05$) but very small amount of regeneration in hosts and grafts.

The principal experiments described in this paper, with their results, are diagrammatically represented in Figure 2.

DISCUSSION

The results reported above are pertinent to four problems, namely: (a) The regenerative ability of animals at different stages of maturity. (b) The origin of differences in regenerative ability; that is, whether they are attributable to variations in the secretory activity of the supraesophageal ganglion or to the relative competence of the tissues. (c) The possibility that there is feedback mechanism involved, whereby maturation is both influenced by, and exerts an influence on, the secretory activities of the brain. (d) The relationship of the "regeneration hormone" and the "juvenile hormone."

When the maximum oöcyte diameter exceeds $140\,\mu$, there is a sharp decline in the ability of the animal to engage in regenerative growth. Mature animals containing fertile oöcytes at least $180\,\mu$ in diameter (Clark and Ruston, 1963a) are incapable of regenerating segments, though a small but complete pygidium is usually formed in each case. This conclusion is consistent with those drawn by Clark and Ruston (1963), Clark and Scully (1964) and Scully (1964).

The second problem was the subject of investigations by Clark and Ruston (1963b) and Scully (1964). They attributed the inability of older animals to regenerate to a virtual cessation of the secretory activities of the supraesophageal ganglion. The second experiment described above demonstrated that the ganglion does indeed decline in potency as maturation proceeds. However, it is still capable of secreting a significant amount of regeneration hormone (at least when implanted into an immature host). The donors used in this experiment contained oöcytes at least $180\,\mu$ in diameter. All were tested, with their ganglia *in situ,* with respect to their regenerative capacity, and found to lack the ability to regenerate segments.

Clark and Ruston (1963b) and Scully (1964) also asserted that the inability of older animals to regenerate is not due to incompetence on the part of the tissues to respond to the hormone. They reported that animals which are unable to regenerate normally will do so if ganglia from immature donors are implanted into them. The number of segments regenerated was small in the experiments of Clark and Ruston (1963b)—only 3 animals produced more than one pair of parapodial rudiments. Scully (1964) obtained the regeneration of many segments. However, though the hosts were at least 90 segments long and control groups failed to regenerate, oöcyte diameters were not determined.

In the experiments reported above, only animals with oöcytes at least 180 μ in diameter were used as "mature hosts." Control groups were tested and found to be incapable of regeneration. Such hosts fail to regenerate significant numbers of segments after receiving implants of ganglia excised from immature worms. The latter (the actual specimens, not a control group) were tested and found to be capable of prolific segment regeneration before the ganglia were transplanted into the mature hosts.

Since ganglia from mature donors induce regeneration in immature hosts, whereas ganglia from immature donors do not cause mature hosts to regenerate, one might conclude that regeneration hormone is secreted in mature *Nereis* but that they are incapable of regenerating because of a deficiency on the part of the body. However, the results do not justify such a conclusion since the experiments involving implantation of ganglia provide no information about the effect of transplantation on the secretory activities of the ganglia. Though a ganglion from a mature animal secretes after implantation into an immature host, it may be inactive in the mature donor. Similarly, though a ganglion of an immature donor is demonstrably active in such an animal, it may be inhibited by transplantation into a mature host.

Comparison of the results obtained from two of the experiments indicates that the mature body may exert an influence on the secretory activities of the ganglion. The significant amount of regeneration ensuing when the ganglion of a mature worm is transplanted into an immature, decerebrate, tail-less host is in sharp contrast to the virtual absence of regeneration occurring in immature grafts implanted into mature hosts. Such grafts do not appear to be subjected to any hormonal influence whatsoever. It is possible that there is a feedback from the maturing animal to the ganglion, inhibiting the secretion of regeneration hormone. Such an influence might emanate from the ripening gametes, the neurosecretory cells of the ventral nerve cord, or from some other source.

A feedback mechanism affecting juvenile hormone secretion in *Perinereis cultrifera* was postulated by Durchon (1952) though his claim that the injection of mature öocytes into immature specimens precipitates maturation has not been substantiated. However, a comparable mechanism affecting the secretion of a hormone promoting gametocyte proliferation in *Arenicola* has been demonstrated by Howie and McClenaghan (1965).

Nevertheless, Hauenschild and Fischer (1962) are clearly correct in their view that the secretory activity of the ganglion is, to some extent, autonomous of the rest of the body. This is shown by the comparatively few segments induced in immature hosts by ganglia from mature donors. The amount of regeneration induced in mature hosts and their immature grafts by implanting several ganglia from immature donors is significant but very small. In comparison, immature grafts in large but immature hosts regenerate an average of 7–8 segments (Golding, 1967b)—without the addition of supernumerary ganglia. This probably indicates that the secretory activities of immature ganglia are not immediately, or completely, inhibited by the *"milieu"* of the mature host.

The fourth problem concerns the relationship of the regeneration-promoting hormone and the maturation-inhibiting hormone. Ruston (1964) thought that two distinct agents are likely to be involved in controlling the processes of regeneration and maturation because of the dissimilarity of the two processes. Clark and Ruston (1963b) investigated the problem and concluded that the two influences are not mediated by a single hormone since they found that oöcyte growth is not inhibited during regeneration, though Hauenschild (1966) reported that maturation is delayed after the loss of many segments in *Platynereis dumerilii.* Their reasoning depended on the assumption that regeneration hormone is secreted either after segment loss but not before, or in greater concentrations after segment loss. However, if the concentration of hormone remains steady throughout regeneration, as has been suggested by Golding (1967b), their results do not demonstrate the existence of two hormones.

The results obtained in this investigation are consistent with the idea that a single hormone influences both regeneration and maturation. The decline in the potency of the ganglion with respect to regeneration occurs mainly after the oöcytes have grown to 140 μ in diameter. Clark and Ruston (1963a) showed that decerebration causes little growth in oöcytes more than 140 μ in diameter in contrast to that induced in smaller oöcytes. From this it appears that the ganglion becomes less effective in promoting regeneration and inhibiting maturation at about the same stage in development.

It may be concluded that one hormone may be responsible for promoting growth (in intact and regenerating animals) and inhibiting maturation. This single hormone would ensure minimum competition between these two vital processes, stimulating growth but holding back maturation in the young animal. Upon its withdrawal, growth would cease and maturation would be precipitated.

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SUMMARY

Normal and regenerative growth are partially separated from maturation, since during the later stages of maturation, growth does not occur. A mature animal almost invariably regenerates a pygidium but rarely regenerates segmental rudiments. The supraesophageal ganglion of a mature animal secretes less hormone than that of an immature animal, though it still induces a significant amount of regeneration when implanted into an immature, decerebrate host. The rate of secretion of such a ganglion is as great during the first five days after implantation into the host as it is subsequently. A single ganglion from an immature donor (in which it is known to be actively secreting) induces no regeneration when implanted into a mature host. Immature grafts, from which posterior segments have been removed, engage in virtually no segment regeneration when stitched into mature hosts. However, implantation of three ganglia removed from immature donors into each graft results in the formation of a significant but very small number of segmental rudiments in host and graft. These results suggest that there may be

a feedback from the maturing body, inhibiting the secretory activity of the ganglion. They are consistent with the suggestion that a single hormone secreted by the supraesophageal ganglion in immature *Nereis* both inhibits maturation and promotes growth in intact and regenerating animals.

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GAMETOGENESIS DURING THE ANNUAL REPRODUCTIVE CYCLE IN A CIDAROID SEA URCHIN (STYLOCIDARIS AFFINIS)[1]

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Within the Echinoidea, the cellular events of gametogenesis have been described in detail for several species: *Echinocardium cordatum* (Caullery, 1925), *Mespilia globulus* (Tennent and Ito, 1941), *Diadema setosum* (Yoshida, 1952), *Strongylocentrotus nudus* and *Strongylocentrotus intermedius* (Fuji, 1960), *Strongylocentrotus purpuratus* (Holland and Giese, 1965) and *Sterechinus neumayeri* (Pearse and Giese, 1966). All of these species, in the presently accepted echinoid classification, belong to the subclass Euechinoidea, and to date there has been no detailed investigation of gametogenesis in any species of the echinoid subclass Perischoechinoidea. The purpose of the present investigation is to describe the cellular events of gametogenesis in *Stylocidaris affinis* (Philippi), a member of the order Cidaroida of the subclass Perischoechinoidea. In addition to occupying an interesting taxonomic and phylogenetic position, the cidaroid sea urchins investigated were living in an environment in which several physical factors had been investigated locally (Hapgood, 1960); this permitted a discussion of possible exogenous control over the annual course of gametogenesis, a topic recently reviewed for echinoderms in general by Boolootian (1966).

Materials and Methods

Urchins were collected by dredge from a population living in about 70 meters of water in Bocca Piccola, the strait between the Isle of Capri and the tip of the Sorrentine Peninsula. Twelve to 17 adult urchins, each weighing between 10 and 40 g., were taken for each sample. In the ten samples taken, the distribution of male and female urchins was as follows: 9 March 1965 (10 M + 6 F); 27 August 1965 (8 M + 5 F); 5 October 1965 (5 M + 7 F); 9 December 1965 (8 M + 5 F); 31 January 1966 (9 M + 3 F); 9 March 1966 (6 M + 8 F); 20 April 1966 (11 M + 6 F); 23 May 1966 (8 M + 7 F); 1 July 1966 (9 M + 6 F); and 2 August 1966 (6 M + 7 F). From each urchin one of the five gonads was removed on the day of collection and fixed overnight in sea water-Bouin's fluid. Fixed gonads were dehydrated in ethanol, cleared in xylene, imbedded in paraffin and sectioned at 7 microns. The gonad sections were stained with haematoxylin and eosin, alcian blue, azure A, periodic acid-Schiff (PAS) or mercuric bromphenol blue by the methods given on page 284 of Holland and Nimitz (1964).

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Oogenesis in female specimens of *S. affinis* was studied by the frequency polygon method of Pearse (1965, page 53). The frequency of different sizes of primary oocytes in the sectioned ovaries stained with haematoxylin and eosin was estimated by measuring at random the diameters of 50 primary oocytes per animal. To be counted as a primary oocyte, a germinal cell had to be sectioned through its nucleolus and had to be at least 11 microns in diameter. Since most such cells were somewhat elliptical in outline, the diameter was always calculated by adding the long and short axes of the ellipse and dividing by two. The germinal cells less than 11 microns in diameter apparently comprised the oogonia and the smallest primary oocytes; such cells could not be counted reliably, since some of them lacked well defined nucleoli and the two cell types were difficult to tell apart. Although the transition of oogonia into primary oocytes is an important part of oogenesis, the present investigation was unable to determine when during the annual reproductive cycle new primary oocytes were produced from oogonia. In the one animal with ripe eggs present in the ovary, the germinal cells were quantified by measuring at random the diameters of a total of 50 primary oocytes (larger than 11 microns and sectioned through the nucleolus) and eggs (sectioned through the nucleus). For each animal, the range of germinal cell diameters from 11 to 110 microns was divided into nine groups at 11-micron intervals and the percentage of cells in each group was calculated. The frequency of occurrence of all size groups in each female was then plotted as a frequency polygon. Also, average frequency polygons were constructed by averaging data from all females in a sample.

Spermatogenesis in the male specimens of *S. affinis* was studied by measuring the thickness of the mass of germinal cells along the wall of testicular lobes that had been cut in approximate cross-section. The type of germinal cell being measured was recorded as spermatocyte, spermatid or spermatozoan. When no spermatids or spermatozoa were present in the germinal cell layer measured, the inner edge of the spermatocyte layer was always sharply demarcated from the central region of the testicular lobe, which was usually filled with non-germinal cells. However, in those testicular lobes containing spermatids or spermatozoa, the entire central region of the lobe was filled with the germinal cells. In such lobes, where the germinal cells did not form an actual layer, the thickness of the germinal cell layer was arbitrarily taken as half of the total diameter of the testicular lobe. In the testes of most male urchins, measurement of a single testicular lobe sufficed to describe the spermatogenic stage in all the lobes. However, in the testes of a few male urchins, some lobes were in one stage of spermatogenesis while other lobes of the same testes were in a second stage; for such testes, it was necessary to measure lobes of each sort and to record the relative abundance of the two types of lobe.

RESULTS

In *S. affinis*, each of the five gonads is essentially an axial gonoduct from which spring elongate, more or less ramified lobes. A histological section through a gonadal lobe reveals an outer visceral peritoneum, a middle connective tissue-muscle layer and an inner layer of germinal as well as non-germinal cells. Within each of the nine samples collected between August, 1965 and August, 1966, the cytometric data from the ovaries were averaged and expressed as nine average fre-

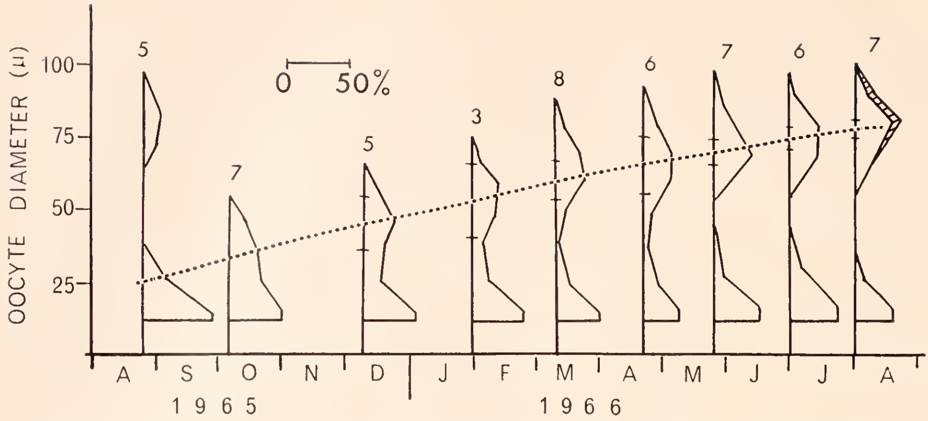

FIGURE 1. Polygons showing the frequencies (see scale) of primary oocyte diameters in the ovaries of *S. affinis* averaged from samples collected from August, 1965 to August, 1966. Each of the nine average frequency polygons was constructed from cytometric data from the number of animals indicated above the polygon. The mean diameter (plus or minus one standard deviation) of the group of growing primary oocytes has been included for most of the polygons. The hatched area of the polygon at the far right indicates ripe eggs. The dotted line drawn through these means describes the progressive increase in the diameter of the group of growing primary oocytes.

quency polygons. By plotting these average polygons, the yearly pattern of oogenesis could be visualized (Fig. 1). As the figure demonstrates, many small primary oocytes began to grow during the first part of September and at no other time during the annual reproductive cycle. In subsequent months, while the growing primary oocytes increased in diameter, numerous small primary oocytes, apparently not growing, continued to be present. Thus, two distinct size classes of primary oocytes could be detected in the ovaries throughout most of the annual reproductive cycle. The curve in Figure 1, drawn through the estimated average diameter of each sample's group of growing primary oocytes, demonstrates that their increase in diameter was not linear with time. It can be calculated, however, that the average volume increase between early December and early August was approximately linear. During this period, the average growing primary oocyte increased in volume at a steady rate of about 200 cubic microns per day.

From Figure 1, it may be seen that the growing primary oocytes attained maximum size and disappeared from the ovaries during the period from early August to late September. The details of this disappearance of the grown primary oocytes from the ovaries may best be visualized by presenting the frequency polygons for each individual female urchin collected in the samples of August, 1965 and August, 1966 (Fig. 2). Figure 2 shows that the ovaries from six of the seven female urchins collected on 2 August 1966 each contained a group of small primary oocytes and a group of grown primary oocytes. The grown primary oocytes had an average diameter of about 80 microns and each cell had a nucleus 35 to 40 microns in diameter containing a nucleolus about 8 microns in diameter. The ovary taken from the seventh female contained a group of small primary oocytes and a group of large germinal cells, more than 90% of which were ripe

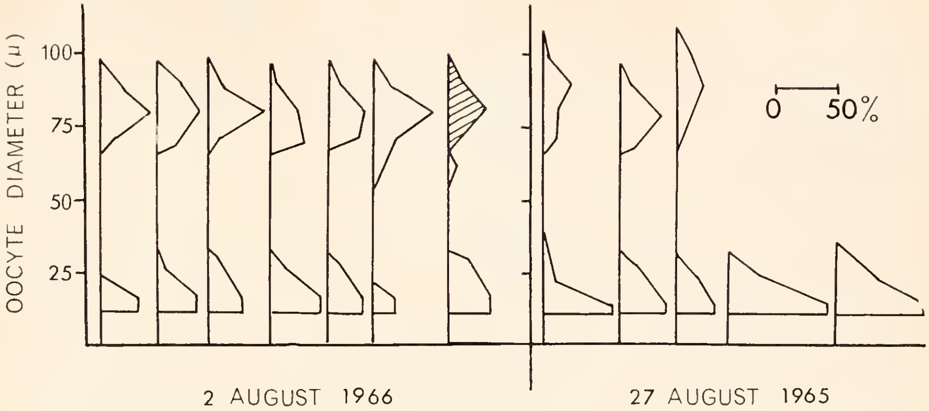

FIGURE 2. Polygons showing the frequencies (see scale) of germinal cell diameters in the ovaries of all female specimens of *S. affinis* collected in August of 1965 and 1966. Unhatched areas indicate primary oocytes; the hatched area indicates ripe eggs. In the sample of 2 August 1966, one of seven females had ripe eggs instead of grown primary oocytes. In the sample of 27 August 1965, three of five females had grown oocytes, while the other two females were devoid of all large germinal cells, presumably because spawning had taken place before collection.

eggs; the few primary oocytes belonging to this group of large cells were its smallest members. On the average, a ripe egg was about 80 microns in diameter and contained an anucleolate nucleus about 14 microns in diameter. In three out of five female urchins collected on 27 August 1965, the ovaries contained a group of small primary oocytes and a group of grown primary oocytes. In the other two females of the sample, the ovaries contained only small primary oocytes and were completely devoid of large primary oocytes or ripe eggs. The most reasonable interpretation of these findings is that the grown primary oocytes matured in the ovary, probably *en masse,* becoming secondary oocytes and then ripe eggs in rapid succession. The ripe eggs were then spawned after being retained in the ovary for a relatively short time (perhaps only a few hours). In the present investigation, the number of samples was insufficient to give precise information about the time elapsing between maturation and spawning. A photographic summary of oogenesis during the annual reproductive cycle is given in Figure 3 A–C.

In sections of ovaries stained with haematoxylin and eosin, all primary oocytes with a diameter of from 11 to 20 microns, as well as the germinal cells smaller than 11 microns in diameter (the smallest primary oocytes and oogonia), had a weakly basophilic cytoplasm, often organized into a loose fibrous meshwork. All primary oocytes larger than 24 microns in diameter had a granular eosinophilic cytoplasm. The primary oocytes ranging in diameter from 20 to 24 microns were in transition between fibrous basophilic cytoplasm and granular eosinophilic cytoplasm. Throughout the annual reproductive cycle, the largest cells in the group of small, non-growing primary oocytes contained cytoplasmic granules. The granular eosinophilic cytoplasm of the primary oocytes was crowded with minute granules of yolk material a fraction of a micron in diameter. These granules stained intensely with PAS after diastase digestion and gave a positive reaction with mercuric bromphenol blue. However, they did not stain with alcian blue

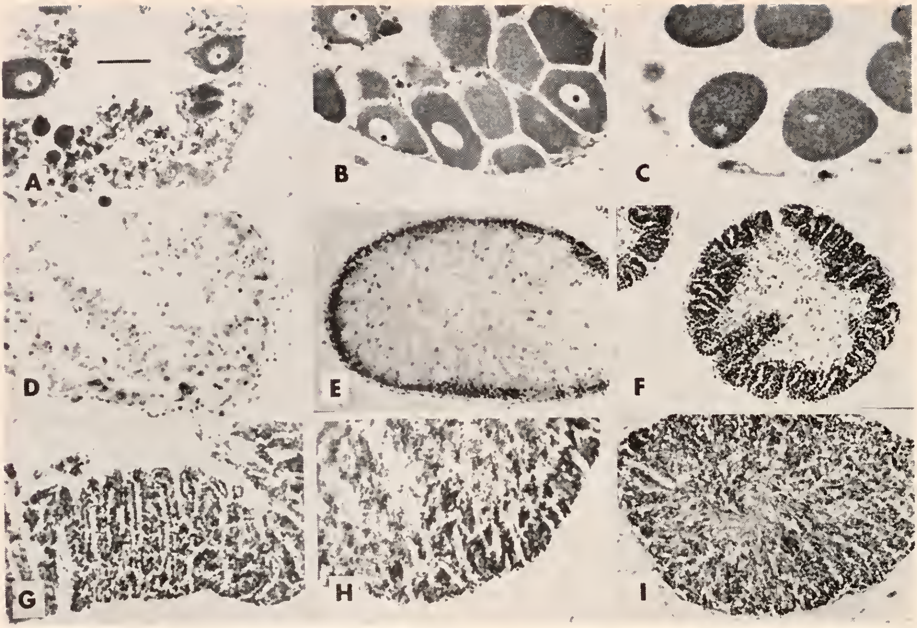

FIGURE 3. Photographs of histologic sections of gonadal lobes of *S. affinis*. Each photograph shows a cross-section of a gonadal lobe or a portion of a lobe with its center toward the top. The ovaries (A–C) were from urchins collected on 9 December 1965 (A), 23 May 1966 (B) and 2 August 1966 (C). In addition to a few small primary oocytes, A and B contain growing primary oocytes. The ripe eggs shown in C are in an ovarian lobe of the only egg-containing specimen of *S. affinis* collected during this investigation. The testes (D–I) were from urchins collected on 5 October 1965 (D), 31 January 1966 (E), 23 May 1966 (F) and 27 August 1966 (G, H and I). In D, there are a few scattered spermatogonia near the edge of the lobe. The next three photographs (E, F and G) show the progressive increase in the spermatocyte layer at the periphery of the lobe. In H (August lobe of type II), the spermatocytes nearer the center of the lobe have given rise to spermatids. In I (August lobe of type IV), the lobe is filled with spermatozoa. All the photographs are shown at the same magnification; the scale line in A is 50 microns long.

and failed to stain metachromatically with azure A. These findings suggest that protein and neutral mucopolysaccharide were conspicuous components of the yolk granules; no acid mucopolysaccharides were histochemically demonstrable. In ovaries sampled in August, some of the grown primary oocytes contained a few cytoplasmic flecks of alcian blue-positive material; this may have been an acid mucopolysaccharide precursor of the cortical material of the ripe egg. In the one ovary that contained ripe eggs, their cortical regions were stained with alcian blue and showed beta-metachromasia when stained with azure A. Thus, the egg cortex in *S. affinis* contained acid mucopolysaccharide components which may have been sulfated. The elaboration of these cortical components apparently occurred suddenly when the primary oocytes were approaching their maximum size. The histochemical reactions of the cytoplasmic yolk granules of the ripe egg resembled those of the primary oocytes, although the PAS reaction was somewhat less intense in the egg.

In sections of testes from all urchins sampled, regardless of the time of year, spermatogonia could be found scattered singly or in small groups just within the basement membrane delimiting the connective tissue-muscle layer from the inner layer of the testis. A typical spermatogonium, although irregular in outline, had an average diameter of about nine microns, and its cytoplasm remained almost unstained by haematoxylin and eosin. The nucleus typically had a diameter of about six microns and was relatively vesicular, containing a large amount of nuclear sap and a loosely organized meshwork of threadlike chromatin; a single, small nucleolus was present in most of the spermatogonia. Since they were always scattered, never forming a continuous layer of cells in the testicular lobes, the spermatogonia could not be quantified by the method used for the other types of male germinal cells. However, the constant presence of spermatogonia in all samples of testes is indicated by open circles interpolated into the base line of the bar graph describing the average course of spermatogenesis during the annual reproductive cycle (Fig. 4).

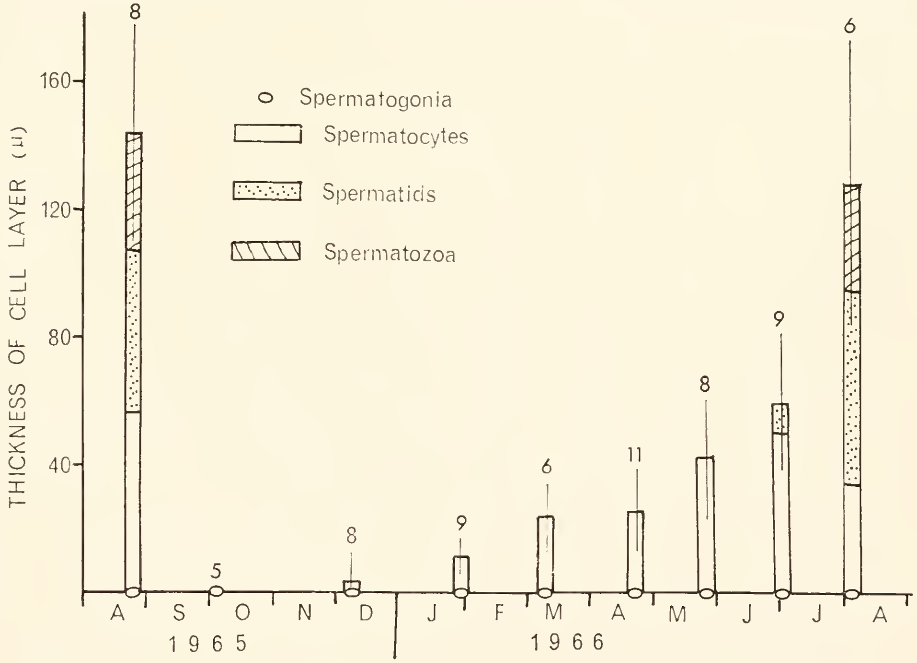

FIGURE 4. A bar graph showing the average thickness and composition of the peripheral layer of germinal cells in the testes of *S. affinis* sampled from August, 1965 to August, 1966. For each of the nine samples, the mean thickness of the germinal cell layer is shown plus or minus one standard deviation. The number of male urchins in each sample is given above each bar; for most samples, only one testicular lobe per animal was measured. However, in some animals sampled in August, two testicular lobes were measured. The open circles interpolated into the base line of the graph indicate the constant presence of spermatogonia. The clear area of each bar indicates spermatocytes, the stippled area spermatids and the hatched area spermatozoa.

The spermatogonia presumably gave rise to more spermatogonia by dividing and also differentiated into spermatocytes. Figure 4 shows that production of spermatocytes began during December and continued until the following August. The basophilic spermatocyte nucleus measured about 4 microns in diameter and was filled with a close-packed tangle of chromatin threads; around the spermatocyte nucleus was a thin shell of cytoplasm, often obscured through crowding by other cells. In the testes of *Stylocidaris affinis* there was no structural intergradation between the spermatogonia and the spermatocytes, as has been reported in the testes of *Strongylocentrotus purpuratus* (Holland and Giese, 1965). In *S. affinis,* the primary spermatocytes apparently could not be distinguished from the secondary spermatocytes. However, it was likely that most of the spermatocytes seen were primary, since the secondary spermatocytes of most animals constitute a very transitory cell type. Throughout the winter, spring and summer the spermatocytes accumulated as an ever-thickening layer of cells. During this extended period of accumulation, the spermatocytes did not differentiate into more advanced germinal cell types. In the sample of 1 July 1966, one out of nine male urchins had spermatids in its testes, heralding the end of the spermatocyte-accumulation phase of spermatogenesis.

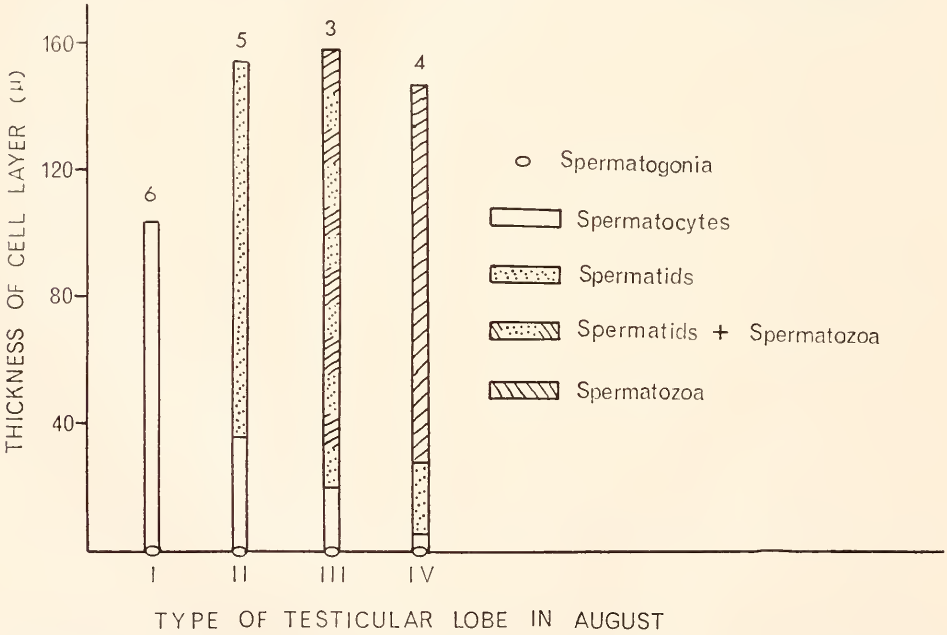

FIGURE 5. A bar graph showing the thickness and composition of the peripheral layer of germinal cells in each of four different types of testicular lobes in the testes of all male urchins sampled in August. The graph is based on data from eighteen testicular lobes from fourteen male urchins collected on 27 August 1965 and 2 August 1966. The respective bars represent an average of six lobes of type I, five lobes of type II, three lobes of type III and four lobes of type IV (the number of lobes averaged is given above each bar). The symbols are the same as in Figure 4.

The testicular lobes of most male urchins sampled in August of 1965 and 1966 contained not only spermatogonia and spermatocytes, but also spermatids and spermatozoa (Fig. 4). Each spermatid had a basophilic nucleus about 2.5 microns in diameter around which no cytoplasm could be demonstrated, while each spermatozoan had a basophilic, pointed conical head, 3.0 microns long and 1.4 microns in maximum diameter; the middle piece and tail were scarcely stained by haematoxylin and eosin. Each primary spermatocyte could give rise to four spermatids and subsequently each spermatid differentiated into a spermatozoan. In general, the spermatids and spermatozoa were less closely packed than the spermatocytes. These factors helped to account for the marked increase in the average thickness of the germinal cell layer of the testes in the August samples. In Figure 4, the bar graphs for the two August samples are averages of four different types of testicular lobes. In the first type of lobe, the layer of germinal cells measured was composed only of spermatocytes. The second type of lobe contained a peripheral layer of spermatocytes and an inner region of spermatids. The third type of lobe contained a peripheral layer of spermatocytes and an inner region of spermatids mixed with spermatozoa (for the calculation of the average bar graphs in Figure 4, this region was arbitrarily assumed to be half spermatids and half spermatozoa). The fourth type of lobe contained a thin peripheral layer of spermatocytes and an inner region of spermatozoa; interposed between the spermatocytes and spermatozoa was a thin layer of spermatids.

For the construction of the bar graphs in Figure 5, data from the testes of the eight male urchins collected 27 August 1965 and the six male urchins collected 2 August 1966 were considered together. Of these fourteen urchins, three had testes with all lobes of the first type, two had testes with all lobes of the second type, two had testes with all lobes of the third type and three had testes with all lobes of the fourth type. The other four urchins had more than one type of lobe in their testes. Of these urchins, three had testes with an average of about 60% of the lobes of the first type and 40% of the second type. The other urchin had a testis with about 80% of the lobes of the third type and 20% of the fourth type. For each of the four lobe types (from six, five, three, and four lobes, respectively), the average thickness and composition of the peripheral germinal cell layer are presented in Figure 5. The most reasonable interpretation of this figure is that the long spermatocyte accumulation stage of spermatogenesis came to an end during the latter part of the summer with a massive differentiation of spermatocytes into spermatids. This spermatocyte differentiation did not necessarily take place simultaneously in all lobes of a given testis, and, on the average, the spermatocytes farthest from the center of the lobe were the last to differentiate. Once produced, the spermatids differentiated into spermatozoa which were probably shed from the testes during the last part of August and the first part of September. The testes of all five male urchins sampled on 5 October 1965 contained no spermatocytes or spermatids and virtually no spermatozoa, with the exception of a few scattered spermatozoa which had been engulfed for digestion by the non-germinal cells (nutritive phagocytes) of the testis. A photographic summary of spermatogenesis throughout the annual reproductive cycle is given in Figure 3 D–I.

In the foregoing descriptions, oogenesis and spermatogenesis have been treated as if the annual reproductive cycle of August, 1965 through August, 1966 follows essentially the same course year after year. This assumption was strengthened by the data from a collection made on 9 March 1965. From the ovaries of six females of this sample, the average frequency polygon of primary oocyte size classes was calculated and found to be virtually congruent with the average frequency polygon of primary oocyte size classes of urchins collected exactly one year later on 9 March 1966. From the testes of the ten males collected on 9 March 1965, the average thickness of the spermatocyte layer was calculated to be 20 microns, and did not differ significantly from the average thickness of 24 microns found exactly one year later on 9 March 1966.

The inner layer of the gonad of *S. affinis* contained, in addition to germinal cells, a population of non-germinal cells (the nutritive phagocytes). In the ovaries and testes, the nutritive phagocytes were similar and may be discussed together. The nutritive phagocytes, each of which had an irregularly shaped nucleus about 5 microns in average diameter, alternated between two morphological phases. They were globulated throughout the fall, winter and spring and they became deglobulated during the summer. In the globulated phase, each cell was typically elongate, measuring about 20 × 12 microns and often containing a voluminous central vacuole surrounded by a shell of cytoplasm. In the cytoplasm there were globules which ranged in diameter from less than 1 to about 10 microns and stained intensely with PAS. The cidaroid's globulated nutritive phagocytes were much like those of *Strongylocentrotus purpuratus;* one such cell is shown in Figure 2 on page 244 of Holland and Giese (1965). In some ovaries there were larger globules, up to 20 microns in diameter, which were probably the remains of broken-down oocytes and eggs. At no time during the annual reproductive cycle did the nutritive phagocytes of *Stylocidaris affinis* become as full of cytoplasmic globules as did those of *Strongylocentrotus purpuratus.* In the male urchins collected on 5 October 1965, the cytoplasm of the nutritive phagocytes contained a few engulfed spermatozoa in addition to globules; these spermatozoa had been left behind in the testis after the majority of the spermatozoa had been shed during August–September. In the testicular lobes with a peripheral layer of spermatocytes, the globular nutritive phagocytes were found internal to the spermatocyte layer. In the ovaries, during much of the reproductive cycle, nutritive phagocytes could be found in the spaces between adjacent primary oocytes as well as in the central part of each ovarian lobe. By the end of summer, in ovaries as well as testes, most nutritive phagocytes had lost their globules and vacuole, and their cytoplasm was extended into long pseudopodial strands. Many deglobulated nutritive phagocytes were mixed with spermatids and spermatozoa in the testes and were found in the spaces between the grown primary oocytes and ripe eggs in the ovaries. It seems likely that some of these non-germinal cells were expelled from the gonads with the ripe gametes at spawning. Presumably, however, enough nutritive phagocytes remained after spawning to repopulate the gonads during the next reproductive cycle.

Discussion

In the last decade, Mortensen's classification of the class Echinoidea (as summarized in Hyman, 1955) has been profoundly revised. Most contemporary students of echinoderm evolution, excepting Philip (1965), have agreed that the former taxa Regularia and Irregularia are to be abandoned and replaced with the subclasses Euechinoidea and Perischoechinoidea. The subclass Euechinoidea contains some fifteen extant orders comprising all irregular echinoids and most regular echinoids, while the subclass Perischoechinoidea contains only one extant order, the order Cidaroida, as can be seen in the diagram on page 369 of Durham (1966). It is generally held that certain members of the order Cidaroida, during the Carboniferous period, gave rise to the ancestral stock of the present-day subclass Euechinoidea, while other members of the order continued in the evolutionary line leading to the present-day order Cidaroida. The cidaroid sea urchins have outlasted all other perischoechinoid orders, which became extinct by the end of the Paleozoic era. The new classification makes living cidaroids phylogenetically more remote from all other living echinoids than previously suspected, and it is of interest to compare selected aspects of gametogenesis in *S. affinis,* in euechinoids and in other echinoderms.

A striking difference between oogenesis in *S. affinis* and the euechinoids which have been studied is the absence in the cidaroid of the extended period of egg accumulation and storage characteristic of the euechinoids. The euechinoid phenomenon appears to be caused by the relatively asynchronous initiation and culmination of growth by the primary oocytes as well as by the prompt maturation of the oocytes on reaching their maximum size. In *S. affinis* as well as in the non-echinoid echinoderms which have been studied, the ovaries never contain ripe eggs for an extended portion of the annual reproductive cycle. Instead, the primary oocytes undergo maturation divisions only a short time before spawning as in *S. affinis* and in the crinoid *Comanthus japonica* (Dan, 1952); or while spawning is in progress as in holothurians (Ohshima, 1925); or a short time after spawning as in asteroids and at least some ophiuroids (Costello *et al.*, 1957). This egg production only at the approximate time of spawning is a widespread, and presumably primitive, characteristic of the phylum Echinodermata, being found in non-echinoid echinoderms as well as in *S. affinis,* where it may represent the retention of an ancient echinoderm trait. By contrast, the extended period of egg accumulation and storage characteristic of euechinoids is an alteration of a presumably primitive echinoderm trait and probably had its origins in the ancestral stock of the subclass Euechinoidea.

Spermatogenesis in *S. affinis* has an extended spermatocyte accumulation phase stretching through much of the annual reproductive cycle; this phase ends abruptly with the production of spermatozoa not long before they are to be shed. In other echinoderms studied, non-echinoids as well as euechinoids, there is no extended phase of spermatocyte accumulation. Instead, since developing germ cells experience no extended arrest as spermatocytes, they continue to differentiate into spermatozoa, and there is an extended stage of accumulation of spermatozoa. Thus, the accumulation of spermatozoa is a widespread, and presumably primitive feature of echinoderm spermatogenesis. The extended phase of spermatocyte accumulation is peculiar to *S. affinis* and is an alteration of a presumably primitive echinoderm trait. In this discussion, I have avoided extrapolation of the findings in *S. affinis* to the order Cidaroida. Clearly, there is a need for detailed studies of more species of cidaroid sea urchins before it is possible to speak of a cidaroid type of gametogenesis.

The population of *S. affinis* sampled was living in Bocca Piccola, one of the straits connecting the Gulf of Naples with the Tyrrhenian Sea. Useful maps of the region of the Gulf of Naples as well as a summary of its geology, meteorology and oceanography can be found in Puri *et al.* (1964) and in Düing (1965). In this region, from January, 1957 to January, 1958, Hapgood (1960) collected oceanographic data once a month at a number of stations, including one in Bocca Piccola (Station D, 75 meters) very close to the sampled urchin population. While Hapgood's data were collected seven years before the present investigation, they are probably a good approximation of the conditions for 1965–1966. The temperature at 75 meters in Bocca Piccola was almost constant throughout the year, averaging 14.3° C.; the lowest reading (13.7° C.) was in early spring and the highest reading (14.8° C.) was in early winter. Oxygen concentrations throughout the year averaged 7.9 mg./liter, fluctuating only slightly from the lowest concentration of 7.4 mg./liter to the highest of 8.4 mg./liter. The average yearly salinity was 37.82‰, and the lowest and highest salinities of the year were 37.58‰ and 38.06‰, respectively. The lack of seasonal fluctuations in temperature, oxygen concentration and salinity indicated that these exogenous factors do not control and synchronize the gametogenesis and spawning of *S. affinis*. On the other hand, the marked annual fluctuations in photoperiod could possibly influence reproductive events of the urchins in Bocca Piccola. The water of Bocca Piccola is clear, having an average annual Secchi disc reading of about 21 meters, and there is sufficient submarine light at 70 meters to support growth of macro-algae. However, the long periods of oocyte growth and spermatocyte accumulation showed no close relation to photoperiod; nor did the short periods of spawning, initiation of oocyte growth, and initiation of spermatocyte accumulation. However, even if light does not have a direct influence on reproduction in the urchins (comparable to photoperiodic induction in some plants), the photoperiod might possibly be used as a periodic reference point to synchronize an endogenous reproductive rhythm, if such exists in the urchin. It is also quite likely that photoperiod could have an important indirect influence on reproduction by influencing the quantity and quality of the algal food available to the urchins. To sum up, the factors (exogenous or endogenous) controlling the clear-cut annual reproductive cycle in this species remain enigmatic. Tortonese (1965) reported that *S. affinis* has a wide geographical distribution (Mediterranean, northwest coast of Africa, Cape Verde Islands, Bermuda, Caribbean and Gulf of Mexico) and a bathymetric range of 30 to 1000 meters. Perhaps a study of gametogenesis in several populations of *S. affinis* living at different depths and in different parts of its geographic range would help shed light on the control of gametogenesis in this species.

The apparent failure of the small primary oocytes to grow in the presence of the growing primary oocytes suggested that the growing oocytes might produce a growth-inhibiting factor to which they themselves were immune but which acted to prevent growth of the small oocytes. On the assumption that lack of growth would be correlated with biosynthetic inactivity, pieces of ovaries of female urchins collected on 23 May 1966 were labeled *in vitro* in H^3-glycine or H^3-uridine for one hour and then prepared for autoradiography. Surprisingly, the autoradiography revealed that there was synthesis of proteins (nuclear and cytoplasmic) and RNAs (nuclear) in both the large and the small primary oocytes. There was certainly no striking suppression of protein or RNA synthesis in the small oocytes; indeed, on the whole, the rate of RNA synthesis in the small primary oocytes appeared higher than in the large primary oocytes. These observations suggest that the small primary oocytes may be growing after all. Perhaps oogonia are giving rise to primary oocytes throughout the year; these primary oocytes subsequently grow to a diameter of 25 to 30 microns and then, if large oocytes are present in the same ovary, break down. This suggestion is supported by the presence in most ovaries of a few small primary oocytes that are apparently deteriorating. If this suggested scheme is indeed the case, the rates of production and destruction of the small primary oocytes would have to be nearly equal to maintain the cell population at the relatively constant size indicated by Figure 1.

SUMMARY

1. Periodic sampling of a Neapolitan population of the cidaroid sea urchin, *Stylocidaris affinis,* revealed an annual reproductive cycle.
2. In female urchins, primary oocyte growth begins only in September and continues for almost a year until maximum size is attained the following August.
3. In the oocytes of this species, conspicuous components of the yolk granules are protein and neutral mucopolysaccharide. Acid mucopolysaccharides, probably destined to be cortical components of the ripe egg, are synthesized only as the primary oocytes are nearing their maximum size.
4. After reaching their maximum size in August, the primary oocytes undergo maturation divisions (probably *en masse*) to become ripe eggs. The ripe eggs are apparently shed soon after being produced in August or September.
5. In male urchins, spermatogonia give rise to spermatocytes, which accumulate in an ever-thickening layer in the testes during the winter, spring and summer. The spermatocytes seem blocked from differentiating into more advanced germinal cell types until late summer, when they abruptly differentiate into spermatids and subsequently spermatozoa. The spermatozoa are apparently shed in late August or early September.
6. The lack of an extended period of egg accumulation in the female urchins, as well as the presence of an extended period of spermatocyte accumulation in the male urchins, sets gametogenesis in this cidaroid (subclass: Perischoechinoidea) apart from gametogenesis in other echinoids that have been studied (subclass: Euechinoidea).
7. The possible control of the annual reproductive cycle by exogenous environmental factors is discussed.

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UTILIZATION OF EXOGENOUS GLUCOSE BY THE REDIAE OF PARORCHIS ACANTHUS (DIGENEA: PHILOPHTHALMIDAE) AND CRYPTOCOTYLE LINGUA (DIGENEA: HETEROPHYIDAE)[1]

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Although the carbohydrate metabolism of some adult parasitic flatworms has been intensively studied (von Brand, 1966; Read, 1961, 1967), the parasitic larval digenetic trematodes have been almost completely neglected. Except for some histochemical observations dealing mainly with the distribution of glycogen, no qualitative or quantitative observations on carbohydrate reserves or carbohydrate metabolism of sporocysts and rediae have been made (Cheng, 1963a; Smyth, 1966). This study was initiated to obtain such information for the rediae of *Parorchis acanthus* (Nicoll, 1906) and *Cryptocotyle lingua* (Creplin, 1825), parasites of marine gastropods.

MATERIALS AND METHODS

Rediae of *P. acanthus* were recovered from *Thais lapillus* and *Urosalpinx cinerea* (see Stunkard and Cable, 1932; Cable and Martin, 1935), and *C. lingua* rediae from *Littorina littorea* (Stunkard, 1930). Naturally-infected snails were collected near Woods Hole by the Marine Biological Laboratory Supply Department and the authors, and maintained in large tanks with a continuous flow of sea water. Cracked *Mytilus* sp. were provided as food for *T. lapillus* and *U. cinerea,* and the *L. littorea* fed on the algal growth on the sides of the tanks. Snails that were used in these experiments were kept in the laboratory for no more than six days although no statistically significant differences were noted in the carbohydrate content of rediae from snails held for as long as two weeks. *Cryptocotyle* rediae from several snails were always pooled for analysis but when possible, *Parorchis* rediae from individual snails were used.

Infected snail hepatopancreas was teased apart in MBL-formula sea water (Cavanaugh, 1964), following the recommendations of Lockwood (1961) that sea water constituted the best experimental medium for tissues from marine animals. Control experiments indicated that rediae remain alive and active in this solution for long periods and that no measurable carbohydrate leakage occurred in two hours. Free rediae were collected with a capillary pipette and washed with three

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changes of sea water. About 200 *P. acanthus* or 500 *C. lingua* rediae were counted into each experimental vessel. Samples were held exactly 30 minutes in an ice bath; excess fluid was drawn off, and 5 ml. of the appropriate medium were added. Snail hepatopancreas experiments utilized tissue slices of about 20 mg. wet weight that were blotted on hard filter paper, weighed on a torsion balance, and placed in 5 ml. of the appropriate medium for incubation. The time interval between each step was held to a minimum.

The media for the various experimental incubations were: (a) MBL-formula sea water (MEDIUM I); (b) MBL sea water containing 0.1, 0.5 or 1.0 m*M* glucose and an isotope (Glucose-U-C^{14}, New England Nuclear) concentration of 0.1 or 0.25 μc./ml. (MEDIUM II); (c) MBL sea water without $NaHCO_3$ and buffered with 10 m*M* tris(hydroxymethyl)aminomethane-maleate plus 0.5 m*M* glucose and 0.25 μc./ml. of isotope (MEDIUM III). In some experiments, phlorizin (Mann Research Laboratories) was added to MEDIUM II at a final concentration of 5×10^{-4} *M*. The gas phases were atmospheric air, 100% N_2, 2% CO_2–98% air, and 2% CO_2–98% N_2.

Incubations were carried out at 20° C. in a controlled temperature water bath for two hours with constant shaking. Experiments in air were done in open 20-ml. beakers, those in the various controlled atmospheres in screw-cap test tubes in which the medium was equilibrated in a manner similar to that described by Dixon (1966).

Following incubation, the tissues were quickly washed onto a polyvinylchloride membrane (Gelman VM-1) in a vacuum filter funnel and flushed with a large volume of sea water. The filter and adhering tissues were placed immediately into a measured volume of 70% ethanol and extracted for at least 12 hours. Aliquots of the alcoholic extract were used for determinations of radioactivity and chemical analysis of "free" carbohydrates.

The alcohol-extracted tissues were washed with 3 changes of 70% ethanol and then digested with 1 N NaOH at 37° C. The amount of protein was determined in diluted aliquots of the NaOH hydrolysate. The remainder of the sample was heated in a boiling water bath for 30 minutes. Alkali-stable polysaccharide was determined in aliquots from this fraction, or alkali-stable, ethanol-precipitable polysaccharide was precipitated with 1.2 volumes of 95% ethanol. The precipitate was washed several times with 70% ethanol containing 0.1% LiCl, and taken up in a measured volume of distilled water. Aliquots were used for determination of radioactivity and analysis of carbohydrate.

Tissue wet weight and initial carbohydrate were determined on rediae held in sea water in an ice bath for 30 minutes. Dry weight was taken on alcohol-extracted tissues which were dried in an oven at 100° C. for 12 hours. Hemolymph was obtained by heart puncture, and blood from several snails was pooled for analysis.

Carbohydrate was determined by the method of Dubois *et al.* (1956). Glucose was specifically identified by the glucose oxidase method with "Glucostat" (Worthington Biochemical Corporation). Reducing compounds were determined by the Nelson method (Nelson, 1944). Protein was measured by the Folin method (Lowry *et al.,* 1951). For determination of radioactivity, 0.1-ml. portions were plated and counted essentially as described by Simmons, Read and Rothman (1960).

Larger experimental samples would have been desirable, but it was not possible to remove more rediae from hosts and separate them into appropriately washed and counted groups in an acceptable time period. The amount of labeled carbon incorporated into 200 to 500 rediae was small, but a series of control experiments confirmed the reliability of the data. These showed that: (1) no significant amount of carbon^{14} was trapped in the filter, (2) negligible amounts of radioactivity remained in the third wash (2 ml. each) from alcohol-extracted tissues, and (3) rediae held for 5 minutes at 60° C. (heat-killed) did not incorporate radiocarbon during a subsequent two-hour incubation.

All results reported are the average values obtained from at least three replicate experiments unless otherwise stated.

RESULTS

Carbohydrate reserves

The physical, chemical and enzymatic determinations made on the trematode and gastropod tissues and fluids are summarized in Table I. Both the rediae and their snail hosts contained relatively large quantities of polysaccharide and glucose plus at least one other freely-extractable, alkali-resistant and non-reducing sugar. The rediae of *C. lingua* were not analyzed in comparable detail because of their small size, but their carbohydrate content per milligram of protein was very similar to that of *P. acanthus* rediae (Tables I, II, III). Rediae of *P. acanthus* recovered from another gastropod, *Urosalpinx cinerea*, were analyzed, and the amounts of extractable carbohydrate (134 $m\mu$Moles/mg. protein) and polysaccharide (1034 $m\mu$Moles/mg. protein) were not significantly different from rediae from *T. lapillus* (Tables I, II).

TABLE I

Physical and chemical determinations

Material	Wet weight (mg.)	Dry weight (mg.)	Prot. (mg.)	NaOH-stable polysaccharide (μMoles)		ETOH-extractable (μMoles)				
						Carbohydrate			Reduc. comp'nds.	
				Non-ppt.	ETOH-ppt.	Total	NaOH boiled	Gluc.	Total	NaOH boiled
Rediae										
P. acanthus[a]	111.5	19.6	15.3	12.86	8.30	1.94	0.59	0.75	3.51	0
C. lingua[b]	—	—	1.0	0.81	—	0.09	—	—	—	—
Hepatopancreas										
T. lapillus	21.8	7.5	4.3	3.44	1.79	—	—	—	—	—
L. littorea	29.3	5.8	2.6	8.57	6.85	—	—	—	—	—
Hemolymph[c]										
T. lapillus	—	—	—	—	—	11.57	9.77	1.79	0.41	0
L. littorea	—	—	—	—	—	12.26	7.47	4.20	0.99	0

[a] About 800 rediae.

[b] About 500 rediae.

[c] Values are μMoles/ml.

TABLE II

Uptake and incorporation of glucose-C^{14} into rediae of Parorchis acanthus. Values are means ± standard deviation.

Medium	Gas phase	No.	ETOH-extractable carbohydrate[a]	Uptake[b]	NaOH-stable polysaccharide[a]	Incorporation[c]
Initial	—	21	143 ± 46	—	1126 ± 260	—
I (pH 7.8)	Air	4	93 ± 8	—	1360 ± 220	—
II (pH 7.8)	Air	10	98 ± 17	8 ± 0.4	1382 ± 146	8 ± 1
II (pH 7.8)	N_2	4	89 ± 15	5 ± 0.5	1296 ± 214	2 ± 0.8
II (pH 6.6)	CO_2/N_2	6	121 ± 46	4 ± 1	1235 ± 126	2 ± 1
II (pH 6.6)	CO_2/Air	3	96 ± 11	0.5 ± 0.4	1172 ± 169	2 ± 0
III (pH 6.6)	Air	3	96 ± 6	0.8 ± 0.3	1029 ± 205	5 ± 1
III (pH 7.8)	Air	3	99 ± 12	1 + 0	1124 ± 77	6 ± 1
III (pH 7.8)	N_2	3	90 ± 3	5 ± 0.5	892 ± 63	1 ± 0.6

[a] Expressed as mμMoles glucose/mg. protein.
[b] Radioactivity as mμMoles glucose/mg. protein/2 hrs.
[c] Radioactivity as mμMoles glucose/μMole glycogen/2 hrs.

Glucose utilization

The amount of carbohydrate from rediae before (*i.e.*, initial values) and after incubation in various media under different gas phases is shown in Tables II and III. The variation in the initial values and in those obtained after incubation in glucose-free medium was great enough to obscure any differences among the experimental samples. In incubations of two hours the true rate of absorption of glucose-

TABLE III

Uptake and incorporation of glucose-C^{14} into rediae of Cryptocotyle lingua. Values are means ± standard deviation.

Medium	Gas phase	No.	ETOH-extractable carbohydrate[a]	Uptake[b]	NaOH-stable polysaccharide[a]	Incorporation[c]
Initial	—	10	121 ± 49	—	696 ± 193	—
I (pH 7.8)	Air	3	76 ± 5	—	1280 ± 87	—
II (pH 7.8)	Air	5	90 ± 29	38 ± 1	1095 ± 75	18 ± 6
II (pH 7.8)	N_2	3	109 ± 36	32 ± 4	1430 ± 232	trace
II (pH 6.8)	CO_2/N_2	3	106 ± 24	20 ± 5	1349 ± 79	trace

[a,b,c] Symbols as in Table II.

C^{14} from the medium is obscured by molecular exchange and metabolism. However, significant differences in incorporation of glucose-C^{14} into polysaccharide under the various experimental conditions were noted.

The rate of incorporation in *P. acanthus* rediae incubated under an atmosphere of N_2 or CO_2/N_2 was about one-fourth the rate of those incubated in air (Table II). That part of this depression of incorporation was due to the lowered levels of oxygen seems likely, but it appears that carbon dioxide also adversely affected the incorporation rate. This is suggested by similar low rates of incorporation in rediae incubated under either CO_2/N_2 or CO_2/air mixtures (Table II). It is difficult to separate the biological effects of carbon dioxide in solution and the resultant change in pH but it appears unlikely that the lowered pH resulting from gassing with carbon dioxide had any marked effect since the incorporation rate of rediae incubated in air at a similar pH (6.6) was only slightly depressed (Table II). The small change in rate observed may have been due to the absence of sodium bicarbonate or to the presence of the tris-maleate buffer in the medium (Medium III) because the incorporation rate of rediae incubated in this medium at pH 7.8 was slightly lower than that of rediae incubated in MBL sea water (Medium II) (Table II).

The results of experiments with rediae of *C. lingua* paralleled those of *P. acanthus.* The rate at which glucose-C^{14} was incorporated into polysaccharide in air was about twice that of *P. acanthus,* and the rate was significantly reduced in atmospheres lacking oxygen and containing carbon dioxide (Table III).

The apparent uptake and incorporation rates of glucose-C^{14} in *P. acanthus* and *C. lingua* rediae were lower in media containing a low concentration of the glycoside, phlorizin (Table IV).

The initial and post-incubation level of carbohydrate and the rate of uptake and incorporation of glucose-C^{14} into polysaccharide by slices of the hepatopancreas of *T. lapillus* and *L. littorea* were determined also. Values were more variable than those obtained for the rediae but incorporation by the hepatopancreas under the different experimental conditions paralleled that of the parasites (Table V). The rate of incorporation in *T. lapillus* tissue more nearly approximated that in *P. acanthus* rediae, while the rate in *L. littorea* more nearly approximated that in *C. lingua* rediae (Tables II, III, V).

TABLE IV

The effect of phlorizin (5×10^{-4} *M*) *on the uptake of glucose-*C^{14} *into rediae. Values are means ± standard deviation where number of experiments permit.*

Species	No.	ETOH-extractable carbohydrate[a]	Uptake[b]	NaOH-stable polysaccharide[a]	Incorporation[c]
P. acanthus					
Control	3	104 ± 74	7 ± 0	1303 ± 212	8 ± 3
Phlorizin	3	104 ± 11	1 ± 0	1224 ± 84	6 ± 0
C. lingua					
Control	2	53	5	1576	13
Phlorizin	3	76 ± 2	3 ± 0	1304 ± 109	9 ± 2

[a,b,c] Symbols as in Table II.

TABLE V

Uptake and incorporation of glucose-C^{14} into hepatopancreas tissue slices. Values are means ± standard deviation where number of experiments permit.

Species	Medium	Gas phase	No.	ETOH-extractable carbohydrate[a]	Uptake[b]	NaOH-stable polysaccharide[a]	Incorporation[c]
T. lapillus	Init.	—	3	199 ± 85	—	469 ± 240	—
	I	Air	3	148 ± 13	—	446 ± 54	—
	II	Air	3	172 ± 35	7 ± 4	678 ± 383	6 ± 2
	II	CO_2/N_2	3	123 ± 23	4 ± 2	363 ± 141	3 ± 0.6
	II	N_2	3	147 ± 30	6 ± 1	463 ± 107	4 ± 1
L. littorea	Init.	—	2	501	—	527	—
	I	Air	2	380	—	338	—
	II	Air	2	366	3	491	20
	II	CO_2/N_2	2	389	1	521	4
	II	N_2	2	407	1	492	4

[a,b,c] Symbols as in Table II.

DISCUSSION

The information on carbohydrate metabolism of trematodes has been subjected to extensive review recently and will not be recounted in any detail here (see von Brand, 1966; Cheng, 1963a; Read, 1961, 1967; Smyth, 1966).

Adult trematodes have a pronounced carbohydrate metabolism, but comparatively little is known about the biochemistry of larval stages. Larval trematodes characteristically contain a substantial carbohydrate reserve which apparently is important in their energy metabolism (von Brand, 1966; Cheng, 1963a), but the meager data available suggest differences in the ability of the various larval stages to utilize this reserve. Thus the miracidium of *Fasciola hepatica* can utilize exogenous substrates and labeled glucose is incorporated into glycolytic intermediates (Bryant and Williams, 1962). *Schistosoma mansoni* cercariae do not develop and emerge from infected snail tissues *in vitro* if adequate quantities of glucose and trehalose are not present in the culture medium (Chernin, 1964). *Cotylurus brevis* cercariae live longer in water to which glucose has been added but they are unable to resynthesize glycogen from glucose (Ginetsinskaya and Dobrovolski, 1963). Certain amino acids prolong the survival of *Fascioloides magna* rediae *in vitro* (Friedl, 1961a, 1961b) and increase the respiration rate of rediae of *Himasthla quissetensis* (Vernberg and Hunter, 1963) but glucose and several other common sugars are without effect in either worm.

The rediae of *P. acanthus* and *C. lingua* contain relatively large quantities of tissue sugars including glucose and are able to take up exogenous glucose and incorporate significant amounts into polysaccharide *in vitro*. Their carbohydrate reserves and glucose utilization rates are comparable to those of some adult parasitic flatworms (see von Brand, 1966).

The importance of carbohydrate in the metabolic activities of mollusks has been well documented (see Martin, 1961; Awapara and Simpson, 1967). It is sufficient to reiterate that the range of blood sugar levels is quite broad both within and between species and that glycogen levels fluctuate under various conditions, *e.g.*, seasonal, nutritional, reproductive. We found *T. lapillus* and *L. littorea* contained considerable amounts of polysaccharide, glucose and other freely-extractable sugars (Table I). In our experiments, the hepatopancreas of each snail absorbed and incorporated glucose at a rate similar to that of the rediae from that host, and incubations under the various gas phases gave parallel results (Tables II, III, V). The similarities are significant if parasite and host tissue are in competition for available glucose.

A considerable portion of the alcohol-soluble carbohydrate recovered from the rediae and from the snails' hemolymph was not glucose and on further analysis was shown to be alkali-stable and non-reducing (Table I). This component may be the disaccharide trehalose, which is widely distributed among invertebrates. Fairbairn (1958) reported this sugar in 71 species representing the major invertebrate phyla, including an adult trematode, *F. hepatica*, and the gastropods, *T. lapillus* and *L. littorea*. Further, the only other free sugar he found was glucose.

In the present experiments, gassing with nitrogen or a carbon dioxide-nitrogen mixture may not have produced absolute anaerobiosis but the oxygen tension must have been reduced to an extremely low level. The marked depression in uptake and incorporation under these oxygen-deficient atmospheres strongly suggests that oxygen is of considerable importance in the metabolism of these rediae and their hosts. There are no data available on the oxygen tensions within the tissues and circulatory systems of the gastropod hosts, but Vernberg (1963) suggests that it is low and variable. All stages in the life cycle of digenetic trematodes utilize oxygen when it is available, but the respiration rate of *H. quissetensis* rediae showed less dependency on reduced oxygen tensions than did any of the other stages of that species (Vernberg, *loc. cit.*).

Our results indicate that carbon dioxide had an inhibitory effect on glucose incorporation that was not attributable to the absence of oxygen nor to the fall in pH produced by carbon dioxide in solution (Table II). In a related study, carbon dioxide stimulated the incorporation of radioglucose into polysaccharide by *C. lingua* adults recovered from gulls (McDaniel, unpublished observations).

It is possible that the adverse effects of oxygen lack and the presence of unlabeled carbon dioxide on radioglucose incorporation in rediae may be related to carbon dioxide fixation mechanisms.

Because of fluxes and metabolism, the values for uptake of glucose-C^{14} from the medium obtained in two-hour incubations are not measures of initial entry rates. However, phlorizin, a glycoside known to be an inhibitor of mediated glucose uptake (Crane, 1960), inhibited the entry of glucose into the rediae (Table IV). It seems possible, therefore, that mediated processes may also be operative in rediae.

Rediae have a simple sac-like gut with a muscular pharynx anteriorly and are able to ingest host cells, but probably there is absorption through the body wall. There is a correlation between phosphatase activity and absorptive function in tissue distribution [although little more can be said about their relationship (see Crane, 1960; Read, 1966)] and alkaline phosphatase activity has been demonstrated in the tegument and in the cells lining the gut of *Echinoparyphium* sp. rediae (Cheng, 1964). The fine structure of the surface of the rediae of *P. acanthus* (Rees, 1966), *C. lingua* (P. Krupa, personal communication) and *F. hepatica* (K. E. Dixon and E. H. Mercer, unpublished observations) is strongly suggestive of an absorptive surface. Mitochondria are numerous in these teguments and "microvilli" increase the surface area available for absorption.

The gut is relatively small in a fully grown redia but with its muscular pharynx does function in the mechanical damage of host tissues (Cheng and Snyder, 1962; Wright, 1966) and frequently contains partly-digested material (Rees, 1966; Cheng, 1963b; Cheng and James, 1960; Krupa, personal communication; Dixon and Mercer, unpublished observations). However, no secretory cells have been found associated with the gut of rediae except for some unicellular glands opening into the esophagus of *P. acanthus* (Rees, 1966). The enzymes responsible for the digestion of the host's tissues may come from the host's cells ruptured mechanically during ingestion.

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SUMMARY

1. The rediae of *P. acanthus* and *C. lingua* and their gastropod hosts, *T. lapillus* and *L. littorea*, contained substantial amounts of "free" carbohydrate and polysaccharide. The free fraction consisted of glucose and at least one other sugar, probably trehalose.

2. Rediae and host heptopancreas tissues absorbed exogenous glucose and incorporated significant amounts into polysaccharide *in vitro*.

3. Glucose absorption in rediae was markedly depressed by a low concentration of phlorizin, an inhibitor of mediated transport systems. Incorporation into polysaccharide was greater in air than under nitrogen, and atmospheres containing carbon dioxide were inhibitory independent of the presence of oxygen.

4. The results support the hypothesis that rediae absorb nutrients through the body surface in addition to ingestion of particulate matter into the gut. The rediae have substantial amounts of glucose available to them *in vivo* and the potential to absorb and utilize glucose has been demonstrated. Since the apparent rates of glucose utilization by parasite and host tissues are similar, the rediae are probably not at a disadvantage in the competition for carbohydrate.

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ASPECTS OF OXYGEN UPTAKE IN MESOCHAETOPTERUS TAYLORI, A TUBE-DWELLING POLYCHAETE[1]

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The genus *Mesochaetopterus* was established by Potts (1914) and now includes five species (Hartman, 1959). The genotype *M. taylori* was collected by Potts at Nanaimo, British Columbia. Subsequent records and observations for this species include those of Berkeley (1922a, 1922b, 1930), MacGinitie and MacGinitie (1949), Ricketts and Calvin (1952), Berkeley and Berkeley (1953) and Barnes (1965). The significance of some of the experiments in the present investigation will be clarified by a brief account of the ecology of this animal. A more comprehensive treatment of ecological aspects, including feeding behavior and tube-building, has been published earlier (Petersen, 1966).

During the autumn of 1965 the authors collected more than 40 specimens of *M. taylori*, at False Bay on San Juan Island in Washington State. At False Bay the animals seem to be restricted to the zone from the middle of the beach to the lowest tidemark. However, it is possible that the population extends into the sub-tidal region as noticed by Potts (1914) at Nanoose Bay, where animals were dredged in 2 to 3 fathoms of water. *M. taylori* inhabits a very long tube, about 0.6 cm. in diameter. The tube projects from 0.5 to 2 cm. above the substrate and extends in a generally straight course to a depth of more than one meter. The tube is composed of an outer layer of sand grains adhering to an inner secreted organic lining. In large tubes the sand grain layer is often inconspicuous and the secreted layer has a parchment-like texture similar to the tube of *Chaetopterus* (Barnes, 1965). Potts (1914) states that a whole undamaged tube was obtained and that it ended blindly in a neatly rounded apex. Fine parchment-like tubes coated externally with sand were secreted by our animals which were kept in glass tubes open at both ends and oriented vertically in sand under running sea water. The lower ends of the secreted tubes were invariably rounded and closed.

In nature the upper end of the tube projects up to 2 cm. above the substrate, a fact that offers protection aganist predation and prevents the entrance of sand and coarse debris into the tube. It will also keep the tube water isolated from trapped surface water at low tide.

Considerable difficulty in digging up intact specimens and tubes has been mentioned by several authors. We attained a certain success by thrusting a spade with a long blade rapidly and vertically into the sand about 6–8 cm. from the protruding end of the tube. After reaching a depth of more than 30 cm. the point of the blade was moved towards the tube to immobilize the animal by compression against the tube wall. The spade with animal and tube was then carefully removed. In the case of broken specimens the remainder of the animal was usually recovered by subsequent digging.

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The experimental part of the present investigation concerns the measurement of oxygen uptake in *M. taylori* at various levels of external oxygen tension. In addition, measurements were made of changes in oxygen availability inside the tube during intertidal exposure. Differences in oxygen tension between interstitial water and tube water were assessed and provided information on the diffusion properties of the tube material. The latter point was also directly tested in the laboratory by changing the oxygen tension across tube wall material while monitoring the rate of oxygen transfer.

Methods and Experimental Procedure

Field sampling

Samples of interstitial water were obtained in close proximity to the tube from which water samples were taken. An aluminum pipe with a cone-shaped end and an inner metal plunger was forced into the sand to the desired depth. By lifting the plunger the interstitial water was drained into the pipe through a series of small holes. Mineral oil applied between the plunger and the pipe prevented contamination of this water by gases in the atmospheric air. A thin stainless steel cannnula was used to sample the water from the enclosed compartment at the end of the pipe. The water samples (0.5 to 1.0 ml.) were obtained at depths ranging from 5 to 30 cm. The samples were analyzed for oxygen tension shortly after they were obtained, using a Beckman Spinco Gas Analyzer (model 160).

Water samples from inside inhabited tubes were taken at 20–40-minute intervals throughout the tidal cycle, starting just before the water subsided and continuing until the water again covered the top of the tubes. These samples were obtained by carefully inserting a thin stainless steel cannula into the tube. After filling the dead space with tube water, the samples (1–2 ml.) were drawn into glass syringes which were subsequently sealed and brought to the laboratory for immediate analysis. The samples were taken from various depths in the tubes (5–30 cm.). All gas analyses were done at 10° C. which corresponded closely with the temperature prevailing in the normal environment.

Laboratory experiments

The oxygen consumption ($\dot{V}O_2$) of *M. taylori* was measured in a closed system using an oxygen electrode as a continuous sensor of the oxygen pressure in the metabolism chamber. The oxygen uptake was determined at various levels of PO_2 as the animals reduced the oxygen pressure in the chamber through their own respiratory activities. The volume of the metabolism chamber was selected in the approximate range of the water volume (30–40 ml.) calculated to be present inside a normal tube. The wet and dry weights of the animals were carefully recorded. Oxygen consumption was expressed as ml. O_2/kg. dry weight/hour.

The diffusibility of oxygen and permeability of water through tube wall material was tested in the following way: A section of an intact tube (15 cm. long) was closed at one end and placed vertically in sea water of 10° C. An oxygen electrode was carefully inserted into the tube compartment and another electrode was placed in the water compartment surrounding the tube. The output from the 2 electrodes was continuously monitored when nitrogen or pure oxygen was bubbled in the surrounding water or in the water phase inside the tube. The rate of change of oxygen tension inside and outside the tube provided the needed information to evaluate the diffusibility of oxygen through the tube wall. The permeability to water was simply assessed by filling one or the other of the compartments described above with sea water and observing any changes in water level.

RESULTS

The interstitial water samples were uniformly low in oxygen tension averaging from 5–8 mm. Hg and invariably less than 12 mm. Hg. No significant relation to depth or phase of outgoing tide was detected. The oxygen tension in the tube water ranged between 70–110 mm. Hg just before the beginning of ebb. Oxygen tension in the surface water covering the sand flats just before exposure during outgoing tide ranged from 130 to 150 mm. Hg, while stagnant trapped pools containing lots of organic material showed a much reduced oxygen tension, at times below 100 mm. Hg. The oxygen tension in tube water declined steadily throughout

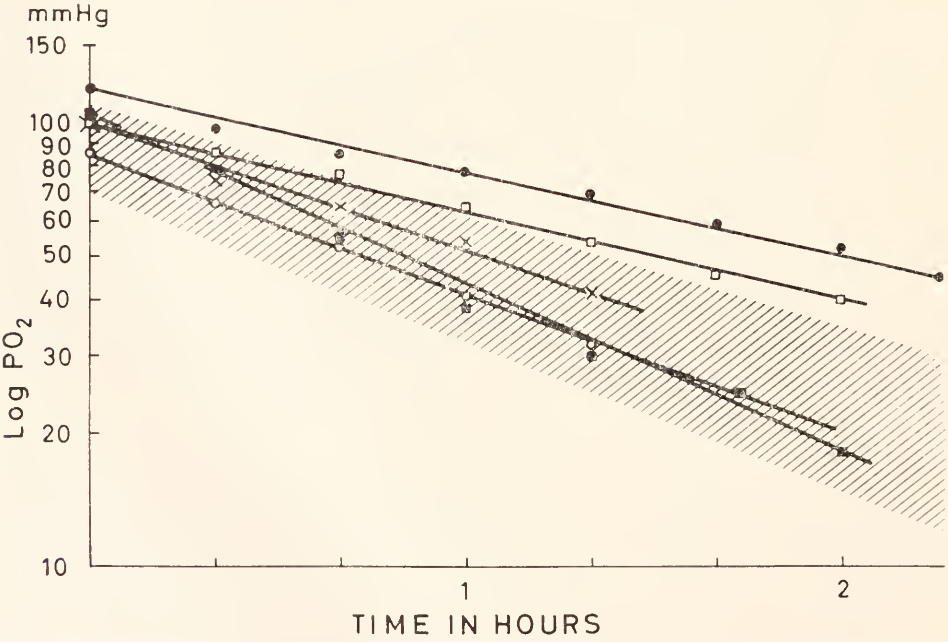

FIGURE 1. Hatched area: Decline of tube water PO_2 during tidal exposure. Start of tidal exposure at time zero. Plotted lines: Rate of PO_2 change in the closed metabolism chambers.

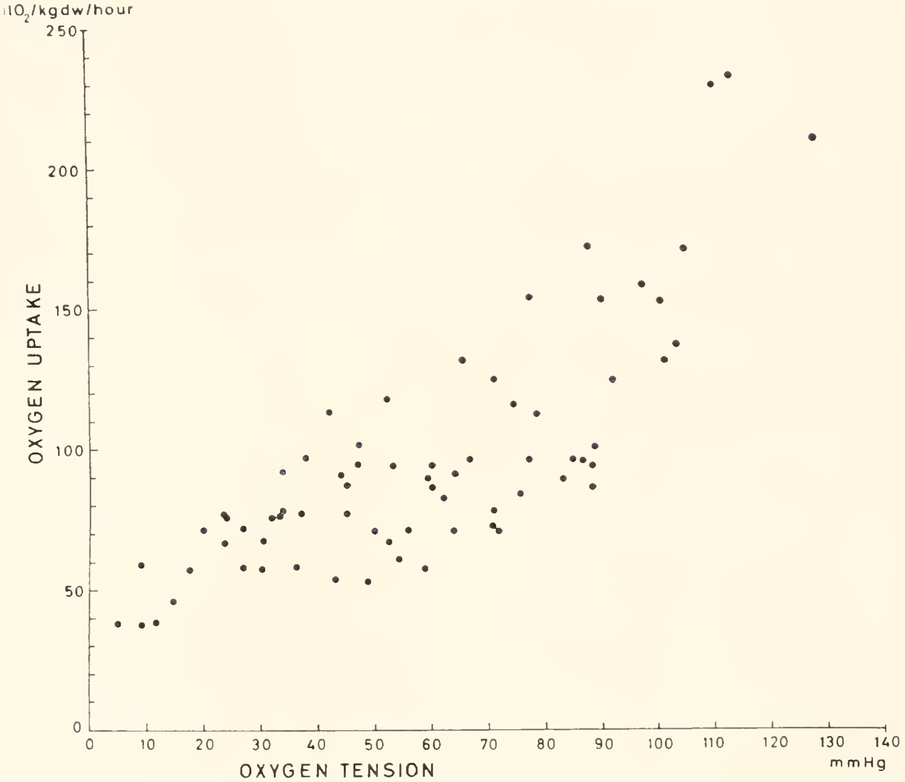

FIGURE 2. Oxygen uptake (ml. O_2/kg. dry weight/hour) plotted against oxygen tension in the ambient water.

the tidal exposure and reached values ranging from 16–30 mm. Hg at the end of exposure periods lasting from 2 to 3 hours.

Figure 1 depicts the average decline in oxygen tension of the tube water (hatched area) plotted as log PO_2 against time. In spite of considerable scatter, due mainly to differences in burrow size and animal size, the values attested to a clear linear regression. The course of the oxygen tension decrease inside the metabolism chambers used in the laboratory experiments (plotted curves) is also shown in Figure 1. Here again there is a set of linear regressions but with varying slopes depending on the size of the animals. The volume of water and animal combined was constant in all experiments and within the range of that occurring in natural tubes. Figure 1 indicates that there must be a linear relationship between oxygen uptake and oxygen tension in the surrounding water. This phenomenon is more specifically expressed in Figure 2 which shows a composite plot of oxygen uptake *versus* oxygen tension in the surrounding water within a range in PO_2 from 110–5 mm. Hg. Figure 1 also points out the importance of the tube wall as a diffusion barrier in as much as the decline of PO_2 was only influenced by the oxygen uptake rate of the animal and not by the steep gradients in oxygen tension shown to be present across the tubes in nature. No significant exchange of gases or water could hence have occurred between the interstitial and tube water compartments. This was substantiated by the experiments designed to directly study the diffusion properties of the tube wall material. These experiments revealed the tube to be virtually impermeable to water and to oxygen within the gradients found to be present under natural conditions.

DISCUSSION

Evaluation of tolerance to oxygen deficiency in intertidal polychaetes requires consideration of a number of factors such as oxygen uptake in relation to external oxygen availability and oxygen tension changes internally in tissues and body fluids. If a respiratory pigment is present, its concentration and respiratory properties, in addition to blood flow and oxygen content of circulating blood, would have to be assessed.

In *M. taylori* there is no respiratory pigment, and survival during tidal exposure will thus depend primarily on the relationship of oxygen consumption to oxygen availability. The present results (Figs. 1 and 2) reveal a steady reduction in oxygen uptake as the surrounding PO_2 goes down. $\dot{V}O_2$ dropped to $\frac{1}{4}$ of the original value when the surrounding PO_2 changed from 100 to 10 mm. Hg. This phenomenon, usually referred to as respiratory dependence, can depend either passively on the reduced external oxygen availability (Van Dam, 1938) or on active changes in the respiratory gas exchange of the animal (Johansen and Vadas, 1967). In order to ascertain whether the latter possibility is operating it is necessary to monitor both external (surrounding water) and internal oxygen tensions simultaneously. Such an approach was unfortunately not technically feasible with *M. taylori.*

The present finding that the PO_2 decline in the metabolism chambers closely resembled those recorded from normal tubes during an average 3-hour tidal exposure indicates that the animals must virtually exhaust their oxygen supply during low tide. The apparent limitation in the distribution of these organisms to the outer region of the intertidal zone may have evolved in response to the limited oxygen supply in the tubes.

On the other hand the results attest to a crucial role of the tube in the survival and distribution of the animal. That is, the tube wall material represents a protective diffusion barrier between the almost anoxic interstitial water and the water inside the tubes. It seems appropriate to emphasize this role as an important survival factor for a sedentary tubiculous polychaete living in an anoxic substrate. The fact that the tube of *M. taylori* is closed at the lower end is essential for the protection offered by the tube wall.

In discussions of adaptive significance related to tubiculous life, protection from predators and possibilities for specialized feeding mechanisms have often been pointed out (Nicol, 1960). However, the biological significance of the diffusion barrier represented by the tube wall, as demonstrated in the present work, appears never to have been appreciated earlier. It would be of interest to know how common this characteristic is among sedentary tubiculous polychaetes. Errant intertidal polychaetes certainly do not enjoy the same protective confinement from the commonly very anoxic conditions of the intertidal substrate (Jones, 1955).

Summary

1. Oxygen uptake, $\dot{V}O_2$, in *M. taylori* has been studied as a function of external oxygen availability. $\dot{V}O_2$ dropped linearly as external PO_2 declined.

2. The rate of PO_2 decline inside normal tubes of *M. taylori* during tidal exposure indicates that the oxygen supply is nearly exhausted during an average 3-hour tidal exposure.

3. A comparison of oxygen tensions in interstitial water and tube water during tidal exposure revealed that the tube is impermeable to water and oxygen. This finding was confirmed by direct laboratory experiments.

4. The distribution and survival of *M. taylori* are discussed in relation to the results obtained.

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PHYSIOLOGY OF THE WHITE CHROMATOPHORES IN THE FIDDLER CRAB, UCA PUGILATOR[1]

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A survey of the literature on chromatophores (Fingerman, 1965) reveals that much more information is available concerning the control of melanophores in the fiddler crab, *Uca pugilator,* than about its white chromatophores. Brown and Sandeen (1948) reported that the white chromatophoric pigment of *Uca pugilator* from the region of Woods Hole, Massachusetts, was more dispersed in animals on a white background than on a black background. The white pigment as well as the melanin of *Uca pugilator* also exhibited a daily rhythm whereby both pigments were more dispersed during the daytime than at night (Brown and Webb, 1948).

Removal of both eyestalks from *Uca pugilator* results in concentration of the melanin (Carlson, 1935); extracts of the sinus glands cause its dispersion (Sandeen, 1950). The white chromatophores respond differently to eyestalk removal; the white pigment becomes maximally dispersed. Furthermore, subsequent injection of extracts of sinus glands did not alter this state in Woods Hole crabs. However, Sandeen did find a high concentration of white pigment-concentrating hormone in the circumesophageal connectives. Because the white chromatophoric pigment of the assay animals used by Sandeen was initially maximally dispersed she could demonstrate only a white pigment-concentrating hormone. She also postulated that an antagonism exists between the melanin-dispersing hormone and the white pigment-concentrating hormone, such that the presence of a large amount of the former decreases the expression of the latter. At that time no evidence was available for the presence of a white pigment-dispersing substance in any crab. Recent studies on *Rhithropanopeus harrisi* (Pautsch *et al.*, 1960), *Carcinus maenas* (Powell, 1962a), *Ocypode platytarsis* (Nagabhushanam and Rao, 1964), *Ocypode macrocera* (Rao, 1967), and *Uca annulipes* (Nagabhushanam and Rao, 1967) have, however, revealed that the white chromatophores in each of these crabs are controlled by two hormones, pigment-concentrating and pigment-dispersing. Therefore, it was decided to reinvestigate the endocrine control of the white chromatophores of *Uca pugilator* to determine whether evidence for a white pigment-dispersing substance could be obtained with this crab also.

To assay for white pigment-dispersing and -concentrating substances it was necessary to obtain two sets of assay animals, one with white pigment in a concentrated state and the other in a maximally dispersed state. In a preliminary experiment it was found that fiddler crabs obtained from Panacea, Florida, would be suitable assay animals. The responses of the white chromatophores of these crabs to light and background were quite different from those reported for Woods Hole crabs by Brown and Sandeen (1948). The experiments described below deal with the (a) daily rhythm of pigment migration in the white chromatophores, (b) responses of the white chromatophores to light and background, (c) endocrine control of the white chromatophores, and (d) antagonism among the substances controlling the black and the white chromatophores of *Uca pugilator* from Panacea, Florida.

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MATERIALS AND METHODS

The animals used in this investigation were specimens of *Uca pugilator* collected in Panacea, Florida, and shipped to New Orleans. In the laboratory the crabs were maintained in stainless steel tanks containing a small amount of artificial sea water. Crabs of 14–17 mm. carapace width were used without regard to sex. At least one day before the crabs were used in an experiment the large chela of the males was removed for convenience in handling them. Eyestalkless crabs which were utilized as assay animals had had their eyestalks ablated at least 12 hours before use.

Extracts of sinus glands, optic ganglia, supraesophageal ganglia, circumesophageal connectives, and thoracic ganglia were prepared in crustacean physiological saline (Pantin, 1934) in the manner described by Sandeen (1950). In addition to preparation of saline extracts, these tissues were extracted with acetone in order to obtain acetone-soluble and acetone-insoluble fractions. The tissue to be fractionated was freshly dissected from the crabs and placed in an embryological watch glass. After preliminary drying at room temperature for 10 minutes the tissue was triturated with a glass rod and extracted with acetone, 1 ml. per organ. The extract was centrifuged for 10 minutes at 1500 *g* and the liquid was decanted into a porcelain evaporating dish and allowed to evaporate. The residue was then extracted in saline to obtain the acetone-soluble fraction. The acetone was free of water when it was first poured on the tissue. The insoluble material was then allowed to dry and extracted with saline, providing the acetone-insoluble fraction.

The dose of each extract injected into an assay animal was 0.05 ml. The extracts were prepared in the following concentrations per dose: one sinus gland, the optic ganglia from one eyestalk, the supraesophageal ganglia from one crab, one circumesophageal connective, and one-half the thoracic ganglia from a single crab.

Each extract was injected into 10 eyestalkless crabs whose white pigment was maximally dispersed and into 10 crabs whose white pigment was maximally concentrated as a result of adaptation for two hours on a black background. The controls, which consisted of eyestalkless crabs and crabs adapted to a black background, received injections of saline in a dose of 0.05 ml./crab. Each experiment was repeated once. All the experiments were conducted during the daytime.

The chromatophores on the walking legs were staged according to the scheme of Hogben and Slome (1931). Stage 1 represents maximal pigment concentration, stage 5 maximal dispersion, and stages 2, 3, and 4 the intermediate conditions.

In order to facilitate comparison of the responses to the several extracts activity values were calculated in the manner described by Sandeen (1950). In each experiment the average stage of the white pigment was recorded at the start of the experiment and 15 and 30 minutes after the extracts had been injected and at 30-minute intervals thereafter for the duration of the response. When pigment dispersion occurs the sum of the average chromatophore stages recorded throughout the experiment for the control group is subtracted from the sum for the experimental group. When pigment concentration occurs the sum for the experimental group is subtracted from the sum of the control group. The differences represent the activity values and constitute a measure of both the intensity and duration of the response.

Experiments and Results

Rhythm of white pigment migration

This experiment was conducted using a group of crabs delivered to the laboratory on March 21, 1967. On that afternoon 40 intact crabs were placed in a plastic container with a small volume of sea water, about 0.5 cm. deep. The container was covered with two layers of black cloth to provide darkness for the crabs. Another lot of 40 was selected and distributed 10 each into two white and two black enameled basins which were kept under a constant illumination of 3.25 meter-candles light intensity. At noon on March 22 the average stage of the white chromatophores of 20 crabs adapted to darkness was determined and the crabs were returned to darkness. The white chromatophores of the crabs on black and white backgrounds were also staged and the crabs returned to their respective backgrounds. This procedure was repeated every four hours through midnight of March 25 and the results are shown in Figure 1. The white pigment of the crabs maintained in constant darkness was more dispersed during the daytime than at night. However, there was no evidence of rhythmical migration of the white pigment of the crabs kept under constant illumination on either background. The white pigment of the crabs on the black background was maximally concentrated while on a white back-

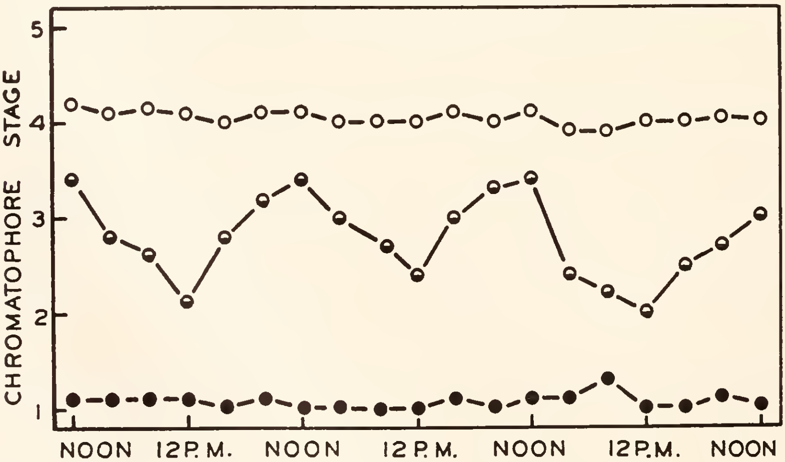

FIGURE 1. Relationships between the stage of the white chromatophores and time of day for crabs maintained in darkness (half-filled circles), in constant light (3.25 meter-candles) on a white background (circles), and in constant light (3.25 meter-candles) on a black background (dots). Observations began at noon of March 22, 1967.

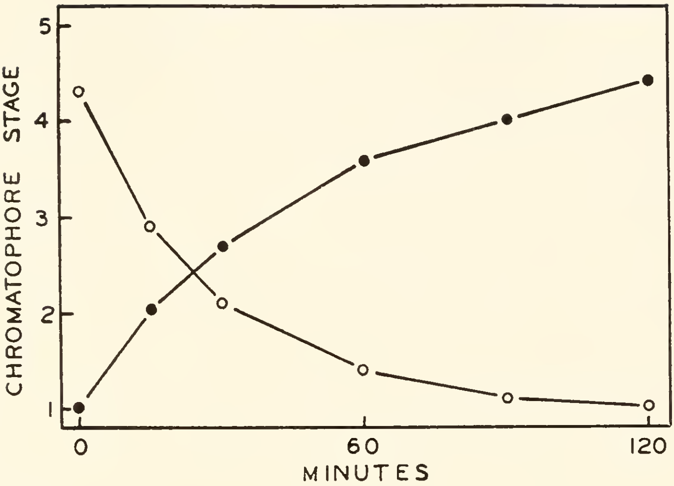

FIGURE 2. Responses of the white chromatophores of *Uca pugilator* to a change of background. Crabs changed from a black background to white (dots), from a white background to black (circles).

ground the pigment was almost maximally dispersed. These results show that in *Uca pugilator* from Panacea, Florida, the background response overrides the daily rhythm at this intensity of incident illumination.

Time required to achieve maximal chromatic adaptation

Twenty specimens of *Uca pugilator* were taken from the stock aquaria and divided into two groups of 10 crabs each. One group was placed in a white enameled basin and the second group in a black enameled basin. At 9 AM both containers were placed under an illumination of 3.25 meter-candles light intensity. At 11 AM the average stage of the white chromatophores in the crabs from each pan was determined. The crabs that had been on a white background were then placed on a black background and *vice versa*. The chromatophore stages of the crabs in each basin were subsequently determined 15, 30, 60, 90 and 120 minutes after the backgrounds had been interchanged. This experiment was repeated once and the averaged data were used in the preparation of Figure 2. As is evident from the figure, the white pigment of crabs on a black background became maximally concentrated. On a white background the white pigment was nearly maximally dispersed. Background adaptation was complete in two hours. The chromatophore stages of the crabs adapted to these backgrounds are essentially the same as seen in Figure 1 for the crabs on the same backgrounds.

Relationships between chromatophore stage and incident light intensity

Ten crabs were placed into each of seven black and seven white basins at 8:30 AM. The crabs in one black and one white container were then exposed for two hours to one of the following intensities of light: 0.19, 0.93, 4.65, 26.0, 52.1, 103.1, and 408.0 meter-candles. Then the white chromatophores of each crab in the 14 basins were staged. This experiment was repeated once. The means of the data obtained from these experiments were used in the preparation of Figure 3. The white pigment of the crabs in black pans remained maximally concentrated at light intensities up to 52.1 meter-candles, but at the higher intensities the pigment dispersed somewhat.

The white pigment of the crabs in the white pan at 0.19 meter-candle light intensity was only dispersed to an intermediate state. As the light intensity increased the degree of dispersion increased to the maximum, stage 5, at 26.0 meter-candles and remained so at all the higher intensities tested.

The next experiment was aimed to determine the relationship between the degree of white pigment dispersion in the chromatophores of eyestalkless Panacea *Uca pugilator* and the intensity of incident illumination. In eyestalkless *Uca pugilator* from Woods Hole the white pigment was in a maximally dispersed state (Sandeen, 1950). In contrast, the white pigment of the Panacea crabs did not respond consistently to eyestalk ablation. Among eyestalkless individuals exposed to a light intensity of 3.25 meter-candles 47% had their white pigment in stage 5, 9% in stage 4, 17% in stage 3, 4% in stage 2, and 23% in stage 1. From a group of eyestalkless crabs 35 individuals with their white pigment in stage 5 and 35 with their white pigment in stage 1 were selected and distributed five each among 14 white enameled basins. One container holding crabs with maximally dispersed white pigment and another with crabs having maximally concentrated white pigment were exposed to one of the light intensities used in the preceding experiment for two hours. Then the chromatophores of each crab in the 14 basins were staged. This experiment was performed three times. The mean chromatophore stages

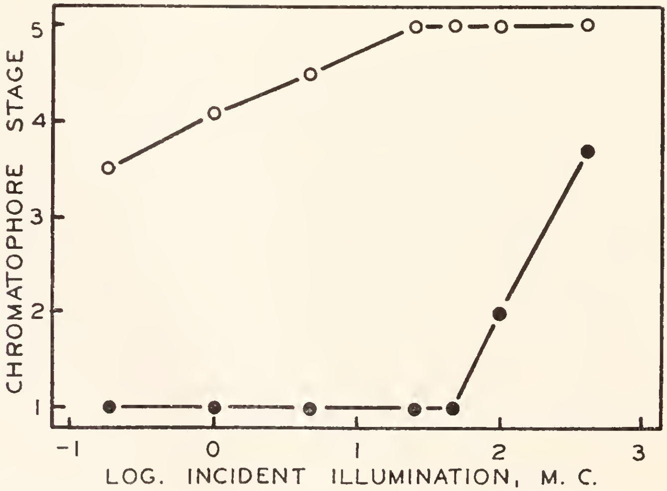

FIGURE 3. Relationships between the stage of the white chromatophores and the logarithm of the incident light intensity in meter-candles for intact crabs during the daytime on a black background (dots) and on a white background (circles).

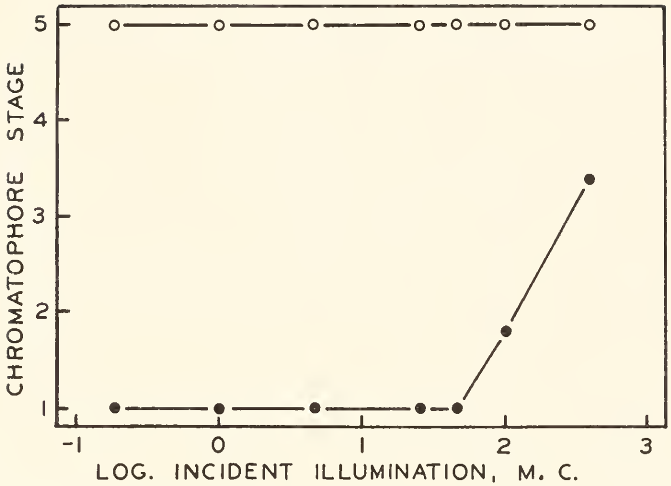

FIGURE 4. Relationships between the stage of the white chromatophores of eyestalkless *Uca pugilator* and the logarithm of the incident light intensity in meter-candles. One group was chosen because its white pigment was maximally concentrated at the low light intensities (dots) while in the other (circles) it was maximally dispersed.

were used in the preparation of Figure 4 where each point represents the average stage of 15 crabs. The degree of pigment dispersion in the chromatophores of the eyestalkless crabs with maximally dispersed white pigment did not change with alteration of the incident light intensity. The concentrated white pigment was also unaffected by light intensities up to 52.1 meter-candles. But at light intensities above 52.1 meter-candles the pigment showed some dispersion with increasing light intensity just as in the intact crabs (Fig. 3) where high light intensities fostered pigment dispersion. This effect is a primary response to light.

Substances controlling the white chromatophores

The aim of this set of experiments was to determine whether or not a pigment-dispersing as well as a pigment-concentrating substance was involved in the control of pigment migration in the white chromatophores of *Uca pugilator.* The activity values for saline extracts of the sinus glands, optic ganglia, supraesophageal ganglia, circumesophageal connectives, and thoracic ganglia injected into eyestalkless *Uca pugilator* and into intact specimens with maximally concentrated white pigment as a result of having kept intact crabs on a black background are shown in Table I. Neither physiological saline nor muscle extracts had any effect whatever on the white pigment whether it was originally concentrated or dispersed.

The saline extracts of the sinus glands, optic ganglia, supraesophageal ganglia, and thoracic ganglia had no effect on the initially dispersed white pigment but did cause dispersion of this pigment. In contrast, the circumesophageal connectives had a pronounced white pigment-concentrating effect but evoked no white pigment-dispersing response.

TABLE I

Activity values for extracts of the sinus glands, central nervous organs, and muscle

	Aqueous extract		Acetone-soluble fraction		Acetone-insoluble fraction	
	Dispersion	Concentration	Dispersion	Concentration	Dispersion	Concentration
Sinus gland	20.0	0.0	0.0	3.4	21.6	0.0
Optic ganglia	18.6	0.0	0.0	7.0	23.4	0.0
Supraesophageal ganglia	13.2	0.0	0.0	0.8	16.8	0.0
Circumesophageal connectives	0.0	12.3	0.0	11.9	0.0	0.0
Thoracic ganglia	13.8	0.0	0.0	6.0	16.0	0.0
Muscle	0.0	0.0	0.0	0.0	0.0	0.0

The acetone-soluble fraction of the sinus glands and central nervous organs evoked in every case at least some white pigment concentration but in no case caused dispersion of the white pigment (Table I). The acetone-insoluble material of the sinus glands, optic ganglia, supraesophageal ganglia, and thoracic ganglia caused no concentration of the white pigment but did cause dispersion of this pigment (Table I). The acetone-insoluble material of the circumesophageal connectives contained neither the white pigment-concentrating nor white pigment-dispersing hormone.

Antagonism between the white pigment-concentrating substance and the white pigment-dispersing substance

The following experiment was devised in consideration of the antagonism that Sandeen (1950) reported between the white pigment-concentrating and melanin-dispersing hormones. Extracts of the supraesophageal ganglia and the circumesophageal connectives from 20 crabs were prepared, each in 1 ml. of physiological saline. One-half ml. of each of these extracts was then diluted with an equal volume of physiological saline. Equal volumes of the two original extracts were then combined to produce a single extract consisting of one-half a complement of the supraesophageal ganglia and circumesophageal connectives per 0.05 ml. Each of the three resulting extracts was injected into 10 eyestalkless crabs and 10 intact crabs with maximally concentrated white pigment. With the eyestalkless crabs melanin-dispersing and white pigment-concentrating activities were determined while with the intact crabs the white pigment-dispersing activity was determined. This experiment was repeated once and the averaged results are shown in Figure 5.

The extracts of the supraesophageal ganglia alone dispersed both the melanin of the eyestalkless crabs (Fig. 5A) and the white pigment of the intact crab on the black background (Fig. 5B) but, as in Table I, did not concentrate the white pigment. The extracts of the circumesophageal connective alone dispersed the melanin and concentrated the white pigment of eyestalkless crabs (Fig. 5C) but, as in Table I, had no effect on the white chromatophores of crabs on a black background (Fig. 5D). The mixture of the supraesophageal ganglia and circumesophageal connectives dispersed the melanin and concentrated the white pigment of eyestalkless crabs

(Fig. 5E) and dispersed the white pigment of intact crabs on a black background (Fig. 5F). The activity values for the three extracts in decreasing order of melanin-dispersing potency are for the supraesophageal ganglia plus the circumesophageal connectives (20.3), supraesophageal ganglia alone (19.2), and circumesophageal connectives alone (9.6). A similar listing for white pigment-dispersing activity is for the supraesophageal ganglia alone (11.2), supraesophageal ganglia plus the circumesophageal connectives (5.8), and circumesophageal connectives alone (0.0). For white pigment-concentrating activity the sequence is circumesophageal connectives alone (11.9), supraesophageal ganglia plus the circumesophageal connectives (2.5), and supraesophageal ganglia alone (0.0). These results demonstrate that when the extracts of circumesophageal connectives and supraesopageal ganglia are mixed the hormones that concentrate and disperse the white pigment are inhibited considerably. The fact that the extract of the circumesophageal connectives produced a melanin-dispersing activity of 9.6 but no dispersion of the white pigment

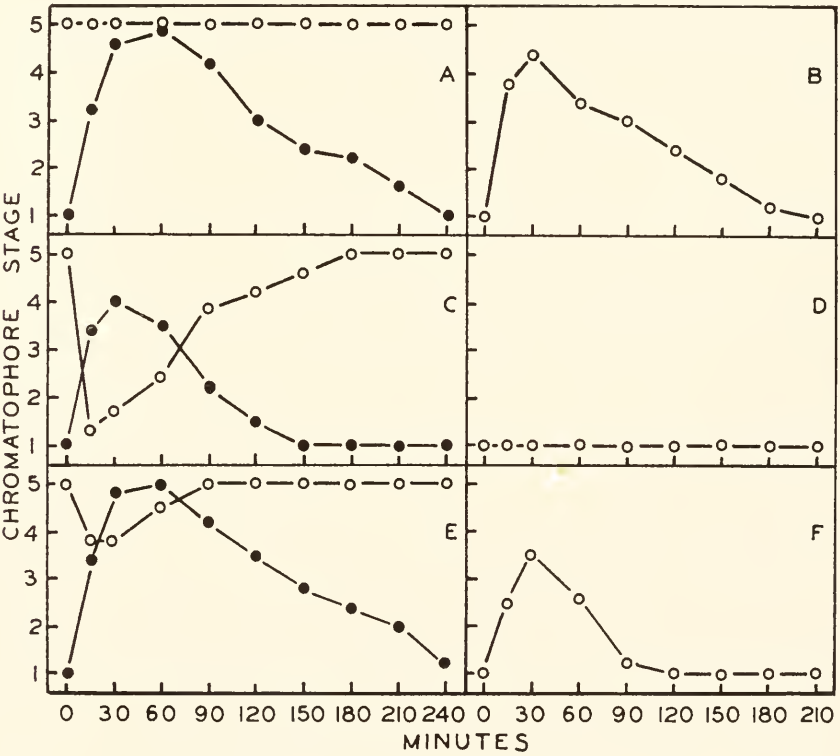

FIGURE 5. Relationships between the stage of the melanophores (dots) and white chromatophores (circles) and time following injection of extracts prepared in physiological saline of the supraesophageal ganglia (A and B), circumesophageal connectives (C and D), and a mixture of equal volumes of these extracts of the supraesophageal ganglia and circumesophageal connectives (E and F) into eyestalkless crabs (A, C, and E) and intact crabs adapted to a black background (B, D, and F). See text for complete explanation.

makes it highly unlikely that dispersion of these two pigments could be due to one hormone. These data will be discussed further below.

DISCUSSION

When the Panacea *Uca pugilator* were maintained in constant darkness the white chromatophoric pigment exhibited a daily rhythm of pigment migration (Fig. 1); the pigment was more dispersed during the daytime than the night. A similar rhythm has been reported for the white chromatophores of *Uca pugilator* from Woods Hole (Brown and Webb, 1948) and *Uca annulipes* (Rao and Nagabhushanam, 1967). However, the amplitude of the rhythm observed for the white pigment of *U. pugilator* from Panacea, Florida, and *U. annulipes* kept in darkness was less than that reported for *U. pugilator* from Woods Hole. The white chromatophoric pigment of *Carcinus maenas* (Powell, 1962b) and *Rhithropanopeus harrisi* (Pautsch *et al.,* 1960) maintained in darkness showed no rhythmicity.

The *Uca pugilator* from Panacea exhibited a pronounced background adaptation. The degree of background adaptation achieved by these individuals was uninfluenced by rhythmicity of the chromatophoric pigment observed in the crabs kept in darkness. In contrast, in Woods Hole *Uca pugilator* the rhythm is a very strong factor in determining the degree of pigment dispersion in the chromatophores of crabs on black and on white backgrounds (Brown and Sandeen, 1948).

The responses to increased illumination of the white chromatophores of *Uca pugilator* from Panacea and Woods Hole were qualitatively alike. In both intact and eyestalkless specimens greater dispersion of the white pigment occurred as the total illumination increased. In contrast, the white pigment of *Uca annulipes* (Rao and Nagabhushanam, 1967) failed to exhibit a true background response; the degree of pigment dispersion was dependent only on the intensity of reflected light.

Of all the extracts prepared in physiological saline only those of the circumesophageal connectives failed to disperse the white pigment in the *Uca pugilator* from Panacea. Sandeen (1950) was unable to determine the existence of the white pigment-dispersing hormone in the *Uca pugilator* from Woods Hole because she used crabs with maximally dispersed white pigment only. Herein evidence is provided for the first time for the presence of a white pigment-dispersing substance in *Uca pugilator.* Although the extracts of the optic ganglia, sinus glands, supraesophageal ganglia, and thoracic ganglia that were prepared in physiological saline provoked white pigment dispersion in *Uca pugilator,* they had no effect on initially dispersed white pigment. However, by using acetone fractionation it was possible to demonstrate the presence of both white pigment-concentrating and -dispersing hormones in all of the organs tested except the circumesophageal connectives. The acetone-soluble fraction of all the tissues had the white pigment-concentrating hormone while the acetone-insoluble fraction of all but the circumesophageal connectives had the white pigment-dispersing hormone. The white pigment-dispersing hormone of *Ocypode* also is insoluble in acetone while the white pigment-concentrating hormone is soluble in this solvent (Nagabhushanam and Rao, 1964; Rao, 1967).

Among the crabs that have been investigated so far the distribution in the nervous system of *Uca pugilator* of the two substances affecting white pigment is unique. The circumesophageal connectives of *Uca pugilator* possess only one of the two substances, the white pigment-concentrating hormone, while the optic ganglia, sinus glands, supraesophageal ganglia, and thoracic ganglia contain both. In contrast, the circumesophageal connectives of *Ocypode platytarsis* (Nagabhushanam and Rao, 1964), *Ocypode macrocera* (Rao, 1967), *Uca annulipes* (Nagabhushanam and Rao, 1967) and *Carcinus maenas* (Powell, 1962a) possess both. In both species of *Ocypode* and *Uca annulipes* the optic ganglia, sinus glands, supraesophageal ganglia, and thoracic ganglia also contain both. In *Rhithropanopeus harrisi,* however, the white pigment-dispersing hormone was found only in the eyestalk (Pautsch *et al.,* 1960), and Powell (1962a) noted that the white pigment-dispersing and -concentrating hormones of *Carcinus maenas* were restricted to the thoracic ganglia and circumesophageal connectives.

As mentioned above, Sandeen (1950) concluded from her experiments that a large quantity of melanin-dispersing hormone decreased the expression of the white pigment-concentrating hormone. When an extract of the circumesophageal connectives was mixed with the extract of supraesophageal ganglia (Fig. 5) the white pigment-dispersing activity of the latter was reduced while the melanin-dispersing activity increased slightly because both tissues contained the melanin-dispersing hormone. In view of the presence in *Uca pugilator* of a white pigment-dispersing hormone, as well as the white pigment-concentrating hormone, a more likely explanation of the antagonism that Sandeen observed is that the antagonism was between the white pigment-dispersing substance and the white pigment-concentrating hormone and that it was merely a coincidence that the extracts she used contained both the melanin-dispersing and white pigment-concentrating hormones.

Although it was shown by the acetone fractionation that the optic ganglia, sinus glands, thoracic ganglia, and supraesophageal ganglia of *Uca pugilator* contain both the white pigment-dispersing and -concentrating hormones, the extracts prepared in physiological saline caused white pigment dispersion only. We could not demonstrate the white pigment-concentrating hormone in the extracts that were prepared directly in physiological saline. If this hormone is present in the saline extracts, then the white pigment-dispersing substance completely inhibited the expression of the white pigment-concentrating hormone. Another possibility is that the latter hormone may be present in the tissues in an inactive (precursor) state, and as such may not be soluble in water. Acetone could act on the precursor liberating an active hormone which is soluble in both acetone and water. If the second possibility is the correct one, then the state in which the white pigment-concentrating hormone occurs in the circumesophageal connectives would have to be different from that in the other parts of the nervous system. It will be recalled that the white pigment-concentrating hormone of the circumesophageal connectives is readily soluble in water (Table I). Moreover, after acetone fractionation of the circumesophageal connectives no increase in white pigment-concentrating activity was observed. In contrast, the presence of white pigment-concentrating hormone in the other tissues was demonstrable only after they were extracted in acetone.

The question was raised above concerning the possibility that the melanin-dispersing hormone and white pigment-dispersing hormone are the same substance and it was concluded from the data of Figure 5 that it is highly unlikely. The fact that the melanin is maximally concentrated in eyestalkless individuals but their white pigment, as mentioned above, was found in all possible stages from maximally concentrated to maximally dispersed also would not be consistent with a unihormonal hypothesis. An intact crab can on occasion even show maximal dispersion of its melanin while its white pigment is maximally concentrated.

The difference between the relative importance of the background response and biological clock in determining the stage of the white pigment of the Panacea and Woods Hole *Uca pugilator* is the second observed difference among these populations with respect to their pigmentary systems. A daily rhythm of melanin migration in both intact and eyestalkless *Uca pugilator* from Florida, has been observed (Fingerman and Yamamoto, 1967), but so far not in Woods Hole fiddler crabs whose eyestalks had merely been removed (Fingerman, Couch and Stool, 1966). Fingerman (1966) has, however, been able to restore the rhythm in eyestalkless fiddler crabs from Woods Hole by implanting sinus glands. Further comparative investigation may reveal more differences between the fiddler crabs of these two populations.

SUMMARY AND CONCLUSIONS

1. Specimens of the fiddler crab, *Uca pugilator,* from Panacea, Florida, exhibited a daily rhythm of migration of their white chromatophoric pigment only when maintained in constant darkness. The pigment was more dispersed by day than at night. Crabs exposed to an incident illumination of 3.25 meter-candles on black and on white backgrounds showed no rhythm.

2. The white pigment of these fiddler crabs exhibited a strong background adaptation. The pigment was well dispersed in crabs on a white background and maximally concentrated in those on a black background.

3. At an incident light intensity of 3.25 meter-candles the white pigment of only 47% of the eyestalkless crabs was maximally dispersed. In 23% of the eyestalkless crabs it was in a maximally concentrated state. High intensities of illumination induced dispersion of the white pigment.

4. Evidence was presented for the first time for the presence of a white pigment-dispersing substance in the sinus glands and central nervous system of *Uca pugilator.* The optic ganglia, sinus glands, supraesophageal ganglia, and thoracic ganglia contain white pigment-dispersing and -concentrating substances. Extracts of these tissues prepared directly in physiological saline revealed only the white pigment-dispersing hormone. However, fractions obtained by acetone extraction of these tissues evoked white pigment concentration while the acetone-insoluble material evoked white pigment dispersion.

5. The circumesophageal connectives are, in contrast, devoid of the white pigment-dispersing substance. They do, however, evoke melanin dispersion in eyestalkless *Uca.*

6. The white pigment-concentrating and -dispersing substances appear to be mutually antagonistic.

7. The question of the possible identity of the melanin-dispersing and white pigment-dispersing substances was discussed. The data suggest that this is a highly unlikely possibility.

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CRYSTAL ORIENTATION IN THE APICAL PLATES OF ABERRANT ECHINOIDS

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The arrangement of skeletal parts in the echinoids is of such regularity that the parts can be assigned an identifying combination of names, numbers and letters. In the regular urchins the periproct enclosing the anus is located at the aboral apex and around it are the five genital plates alternating with the five ocular plates. Although there is some question concerning the premises Lovén (1874) used in setting it up, his system for numbering these plates is now almost universally used by students of these animals. The landmark in the system is the madreporite, a modified genital plate. The genital plates are numbered with Arabic numerals 1 through 5 counterclockwise as one looks down on the apex, with the madreporite assigned number 2. The ocular plates are assigned Roman numerals and precede their genital counterparts in the counterclockwise sequence as shown in figure 1.

In the genus *Strongylocentrotus* it is usual for ocular plates I and V to abut the periproct separating the adjacent genital plates from each other. Such ocular plates are termed "insert" (Jackson, 1912). The bases of the other ocular plates are usually occluded from the periproct by the abutting bases of the genital plates beside them and are called "exsert" according to Jackson's terminology.

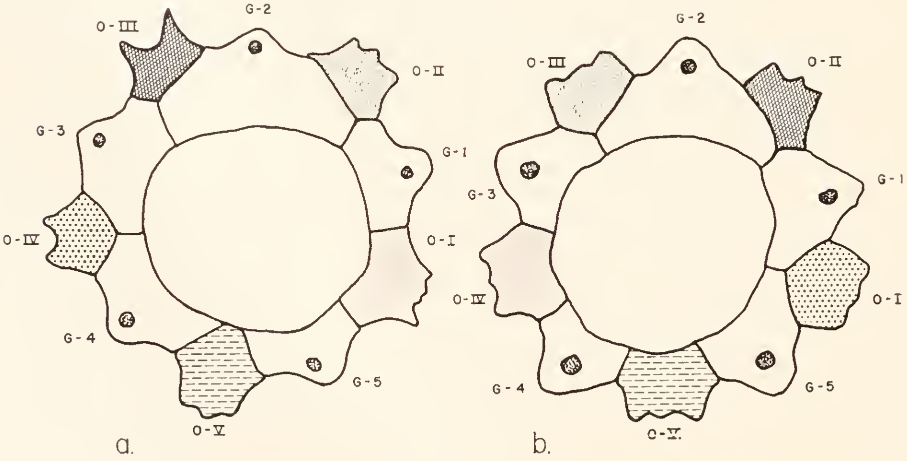

FIGURE 1. Apical system morphology in *Str. franciscanus.* Ocular plates are shaded arbitrarily to emphasize the effects of inversion. *a,* normal specimen (#78). *b, situs inversus* specimen (#81) from same population.

Swan (1966: pp. 414–417 with additional references on p. 434) has summarized the abundant literature on morphologically aberrant individuals. Many of these result from injury followed by imperfect regeneration. Other types of aberrancies appear to be related to deviations from the normal developmental pattern. The most frequently reported categories are (1) departures from pentamerous symmetry, either through the addition or deletion of radial elements, and (2) variants involving the number and individuality of ocular plates insert. Jackson (1927) summarized the records of non-pentamerous echini that had been reported previously, and since then additional cases have been reported—most recently tetramerous specimens of *Meoma ventricosa* and *Encope michelini* (Kier and Grant, 1965). Jackson (1912, 1914) had previously collected a great amount of information on variation in respect to insertion of ocular plates, and Vasseur (1952) and Swan (1962) have added additional data for the genus *Strongylocentrotus.*

Early in the normal development of the *Strongylocentrotus* apical system, all five ocular plates are exsert, *i.e.,* they do not make contact with the inner margin of the oculo-genital ring. During ontogeny, variation in growth rates changes the morphological pattern and one or more ocular plates becomes insert (that is, occupying a position that completely separates the adjacent genital plates). In *Strongylocentrotus,* ocular I is generally the first to become insert. This is followed by ocular V and occasionally by ocular IV and then rarely by ocular II. Ocular III remains exsert. Jackson (1912) classified the variants in relation both to number of ocular plates insert and to which individual plates were involved. Thus in *Strongylocentrotus,* where oculars I and V are normally insert, adult specimens with fewer than two plates insert are called regressive variants, while those with more are called progressive. Regardless of how many ocular plates are insert, if these plates are not in one of the normal ontogenetic arrangements, the individual is an aberrant variant.

Swan (1962) noted that some of the aberrant variants appeared to be mirror images of members of the normal sequence. Such would be the case for specimens with oculars IV; IV and V; or IV, V, I and III insert. Specimens with no oculars insert or with IV, V and I insert may also be mirror images, but are not detectable on this basis. More careful examination of these often revealed that the anus was displaced toward the opposite side from usual, and this can be seen in Jackson's (1912) Text-Figures 140 and 142. Internally such specimens had their digestive tracts coiling in the opposite direction from usual and generally deviated from perfect radial symmetry exactly as a mirror image of the usual condition. Thus they are morphologically examples of *situs inversus.*

A comparison of *situs inversus* and normal specimens is shown in Figure 1. These sketches are traced from thin sections of the articulated apical systems. The ocular pores are generally lost in sectioning, but the plate patterns are otherwise comparable to those observed on the surface of fresh specimens. The ocular plates in Figure 1 have been shaded arbitrarily to emphasize the inversion: O-I of the normal specimen is comparable to O-IV of the inverted specimen and O-IV of the normal specimen is comparable to O-I of the inverted one.

Crystal orientations in the echinoid skeleton are reasonably well known. Data have been published for approximately 250 fossil and living species (Raup, 1966a)

TABLE 1

Specimens of Strongylocentrotus used in crystallographic analysis

Series	Collection date	Collecting locality	Depth	Number	Species	Morphology
Y-1	June 28, 29, 1957	"The Nubble," Cape Neddick, York, Maine	intertidal	242	*dröbachiensis*	normal
				244	*dröbachiensis*	*situs inversus*
				253	*dröbachiensis*	*situs inversus*
				279	*dröbachiensis*	normal
				563	*dröbachiensis*	tetramerous
E-1	August 17, 1958	Edmunds, Maine	intertidal	57	*dröbachiensis*	tetramerous
f-2	July 6–21, 1959	between Cantilever Pier of U. of W. Labs. and Pt. Caution, Friday Harbor, Wash.	0–8 ft.	49	*franciscanus*	normal
				50	*franciscanus*	*situs inversus*
				78	*franciscanus*	normal
				81	*franciscanus*	*situs inversus*
pM-1	August 18, 19, 1959	Makah Bay, Olympic Peninsula, Wash.	intertidal	106	*purpuratus*	normal
				107	*purpuratus*	*situs inversus*
eP-1	August 27, 1959	East of Peavine Pass, San Juan Islands, Wash.	156–168 ft.	146	*pallidus*	normal
				147		*situs inversus*

and the general subject has been reviewed by Raup (1966b). In nearly all situations the individual skeletal part behaves optically as a single crystal of calcite ($CaCO_3$). Furthermore, the crystallographic orientation of the calcite is quite systematic and regular and is apparently not sensitive to local environmental conditions. In crystallographic studies particular attention has been paid to the orientations of crystals in the plates that make up the oculo-genital ring of the

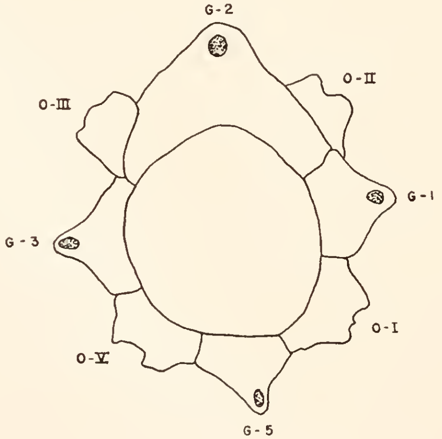

FIGURE 2. Apical system morphology in a tetramerous specimen (#57) of *Str. dröbachiensis.* Plates were identified in part on the basis of crystallographic evidence.

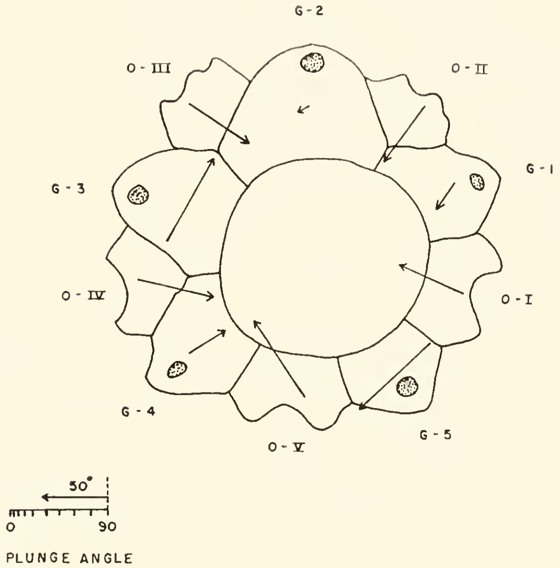

Figure 3. Crystal orientations in the apical system of a normal specimen (#279) of *Str. dröbachiensis.* Arrows indicate the direction of plunge of the *c*-axis. The length of the arrow increases as the angle of plunge decreases, so that the longest arrows are *c*-axes that are nearly tangent to the plate surface.

apical system (Raup, 1965). On the basis of several lines of evidence, it has been shown that crystal orientations in the echinoid apical system are established at the time of metamorphosis and that the orientations of crystals do not change appreciably during development. If the morphological aberrations are established at the time of metamorphosis, then those aberrations should also be reflected in the pattern of crystal orientations.

MATERIAL

Specimens of several species of the echinoid genus *Strongylocentrotus* were used in this study. The sample includes representatives of two prominent types of morphological aberration. The tetramerous aberration is represented in the sample by two specimens from different populations of *Strongylocentrotus dröbachiensis. Situs inversus* is represented in the sample by six specimens distributed among *Strongylocentrotus dröbachiensis, S. franciscanus, S. purpuratus,* and *S. pallidus.* Accompanying each of these specimens is one or more normal individuals of the same species from the same locality to serve as controls. The suite of specimens and their locality data are summarized in Table I.

Figure 2 shows a sketch of a thin section of one of the tetramerous specimens. Only four ocular and four genital plates are present. The plates have been labeled to indicate that O-IV and G-4 are the missing plates. The evidence for this interpretation will be presented in this paper.

METHODS

Thin sections of all specimens used in the study were prepared. The sections were cut in the plane of the apical system and thus included the articulated apical system and in most cases, a few plates of the adjacent ambulacral and interambulacral columns (not shown in Figs. 1 and 2). When the thin sections are viewed in polarized light, the plate boundaries are particularly clear because of the differences in crystal orientation from plate to plate. These differences enhance the morphological distinctness of the plates.

Crystal orientations in each thin section were determined by standard optical methods using a petrographic microscope and universal stage (see Raup, 1960, for general review of the method). The orientation in three dimensions of the principal optic axis (*c*-axis) was determined for each ocular and genital plate. Typical data for a normal specimen are shown in Figure 3. For each plate, an arrow indicates the azimuth of the *c*-axis (projected to the plane of the plate). The *point* on the arrow refers to the direction of the pluge of the *c*-axis. For example, the *c*-axis of ocular II in Figure 3 plunges down toward a point below the center of the apical system. The *length* of the arrow is inversely proportional to the angle of plunge (the angle that the axis makes with the plane of the thin section). By convention, a 0° plunge is a horizontal or tangential *c*-axis; a 90° plunge is a *c*-axis which stands perpendicular to the surface of the plate.

In order to summarize the data from a variety of thin sections, the azimuths have been evaluated numerically, as the angle formed between the *c*-axis (projected to the plane of section) and an imaginary line connecting the center of the plate and the center of the apical system. This system of expressing azimuths is explained in Figure 4. Presentation of azimuth data in this way assumes, of course, a morpho-

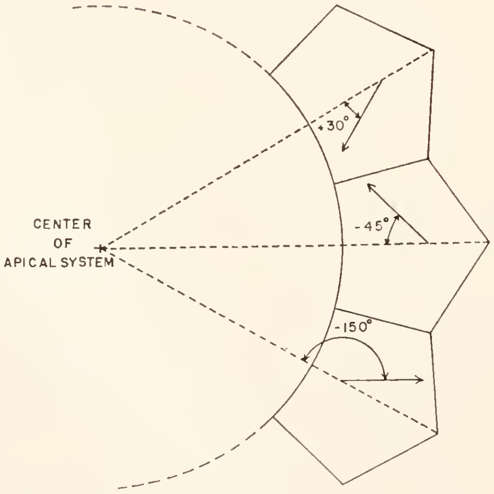

FIGURE 4. Method for expressing azimuths of *c*-axes. A *c*-axis which plunges directly toward the center of the apical system is assigned a 0° azimuth. Those plunging to the right (when viewed from the center of the apical system) are assigned positive azimuths and those plunging to the left are assigned negative azimuths.

logical perfection rarely attained in the echinoid apical system. The apical system is not perfectly circular and thus the selection of the center of the apical system is inevitably subjective. The method is satisfactory for the present purposes, however.

RESULTS

Normal specimens

Figure 3 is typical of the results for specimens with the normal morphology. The pattern of crystal orientations is completely reasonable in terms of previous work on orientation in the echinoid apical system (Raup, 1965). As is typical for the genus, the *c*-axes of genital plates 1, 2 and 4 are nearly perpendicular to their respective plate surfaces. This is indicatd in Figure 3 by the shortness of the arrows for these plates (plunge angles are 72°, 89°, and 66°, respectively). As can be seen from the figure, the *c*-axes of genital plates 3 and 5 are nearly parallel to the plane of the plate. Plunge angles in G-3 and G-5 of *Strongylocentrotus* rarely exceed 10°. Furthermore, these two plates have quite characteristic azimuths in that the *c*-axes tend to parallel the inner margin of the apical system.

The *c*-axes of the five ocular plates in Figure 3 plunge more or less toward the center of the apical system. Plunge angles average about 40°. This is also typical for the genus (Raup, 1965).

Table II gives crystallographic data for all specimens used in this study, including the one illustrated in Figure 3. The azimuths are expressed as explained in Figure 4. The data for the six specimens having normal morphology are in general accord with the generalizations just presented. Note that the azimuth values for genital plates 3 and 5 are all reasonably close to either plus or minus 90° and thus conform to the azimuths of these two plates in Figure 3. The azimuth data for the other three genital plates are quite scattered but this is to be expected because of the high plunge angles (60° or greater).

Also, in Table II, note that the azimuths for ocular plates of normal specimens are all quite close to zero. The greatest departures are found in the orientations for O-IV and O-V.

Situs inversus specimens

The azimuth and plunge data for the six specimens with inverted morphology are also given in Table II. Note that the plunge angles for plates G-1 and G-4 are close to zero and thus are comparable to G-3 and G-5 of the normal specimens. This is what would be expected if the crystallography were inverted. With reference to Figure 1, we can predict which plates in the *situs inversus* forms should be homologous to plates in normal individuals: this has been done in Table III.

Table III gives the predicted correlation beween equivalent plates in the normal and *situs inversus* specimens with average values of the crystallographic data for each from Table II.

The method of calculating average values from the data in Table II requires some explanation. The data for ocular plates present no problem because all *c*-axes plunge approximately toward the center of the apical system. The ocular

Table II

Crystallographic data for echinoid apical systems

	Azimuth:										Plunge:									
	Genital					Ocular					Genital					Ocular				
no.	1	2	3	4	5	I	II	III	IV	V	1	2	3	4	5	I	II	III	IV	V
Normal																				
242	−22	−127	−92	13	−78	7	−11	−3	−17	50	72	72	11	59	8	33	42	28	21	15
279	30	−68	77	−7	111	−8	−3	14	−24	47	72	89	6	66	3	47	45	44	38	19
49	−1	30	−122	−1	83	4	−2	−5	−22	25	64	85	3	60	0	43	42	46	52	54
78	27	−31	69	−22	100	7	−11	3	1	69	60	76	4	78	3	48	34	64	68	37
106	8	−142	88	−5	102	−3	3	2	−2	−5	70	80	4	74	1	32	25	45	35	36
146	−11	−69	−103	28	−84	12	−6	3	6	37	76	68	1	78	7	53	28	53	40	26
Situs inversus																				
244	−67	38	−14	73	11	35	10	7	−4	*	14	79	67	5	74	28	46	15	45	*
253	122	21	4	−87	6	22	−20	24	6	−17	40	60	44	12	67	22	55	45	43	12
50	99	−20	−6	−99	−18	5	5	11	0	−47	8	75	55	8	66	54	45	36	33	42
81	−84	56	−2	88	7	−10	−2	0	10	−36	3	67	66	4	66	52	40	29	33	52
107	120	8	−26	91	29	12	−4	9	−2	7	3	81	63	3	66	37	18	27	19	10
147	100	116	−51	−92	40	31	−6	20	−3	−27	12	70	83	21	80	48	45	42	58	22
Tetramerous																				
563	4	−60	−117	22	−85	0	−23	10		−154	68	69	15	67	5	45	45	42		22
57	−12	−22	−110		96	−8	4	−30		21	46	62	23		0	52	51	56		24

* Crystallography indeterminate.

Table III

Average values for crystallographic data

Normal:		*Situs inversus:*	
Plate	Angle	Angle	Plate
Azimuth			
G-1	5.2	15.8	G-3
G-2	−67.8	−36.5	G-2
G-3	−103.8	−108.3	G-1
G-4	1.0	−12.5	G-5
G-5	−81.0	94.3	G-4
O-I	3.2	−1.2	O-IV
O-II	−5.0	−11.8	O-III
O-III	2.3	2.8	O-II
O-IV	−9.7	−15.8	O-I
O-V	37.2	24.0	O-V
Plunge			
G-1	69.0	63.0	G-3
G-2	78.5	72.0	G-2
G-3	0.2	7.7	G-1
G-4	69.2	69.8	G-5
G-5	1.3	4.8	G-4
O-I	42.7	38.5	O-IV
O-II	36.0	32.3	O-III
O-III	46.7	41.5	O-II
O-IV	42.3	40.2	O-I
O-V	31.2	27.6	O-V

data in Table III are thus simple averages of the corresponding data in Table II except that the signs have been reversed on the azimuths for the *situs inversus* specimens. This change is necessary in order to correct for the inversion (see Fig. 4).

Averaging of the genital plate data is more complex, particularly for those plates where the *c*-axis approximately parallels the inner margin of the apical system (G-3 and G-5 in normal specimens and G-1 and G-4 in *situs inversus* specimens). Consider the data in Table II for G-3 of the six normal specimens. The *c*-axis of this plate in three of the specimens plunges in a counterclockwise direction (negative azimuth) and in the other three specimens plunges in a clockwise direction (positive azimuth). With reference to plunge angle, the counterclockwise plunges slightly outweigh the clockwise plunges and thus the average azimuth should be negative. To achieve this, −180° was added to all positive azimuths for G-3 (normal specimens) before averaging. The same procedure was followed for G-5 of the normal specimens and for G-1 and G-4 of the *situs inversus* specimens. (In the G-1 data for the *situs inversus* specimens, +180° was added to the negative azimuths because the average plunge is clockwise.) Before entry in Table III, signs were reversed for the *situs inversus* specimens in order to correct for the inversion (as was done for the ocular data, above). The data for the remaining genital plates were averaged by the method that was used for the ocular data. In the case of G-2, little confidence should be attached to the average azimuths because of the wide scatter of raw data (stemming from the near −90° plunges).

The correspondence in Table III between the crystal orientations of normal specimens and those of *situs inversus* specimens (corrected for the inversion) indicates that the crystallography is inverted as well as the morphology. This is shown graphically in Figure 5. In this figure, all the data from Table III are plotted on a somewhat generalized sketch of the normal apical system morphology. The solid arrows are the average crystal orientations for the six normal specimens and the dashed arrows are the averages for the six *situs inversus* specimens. The most apparent difference between the two sets of arrows is in plunge direction for G-5. This difference is actually minor, however, because both average plunges are close to zero (see Table III).

Tetramerous specimens

One of the major problems encountered by Jackson and others in working with departures from pentameral symmetry has been that of identifying the skeletal elements that are added or deleted. Because crystal orientations in genital and ocular plates form a distinct pattern, crystallographic evidence may be applied to the problem. Both tetramerous specimens used in this study obviously lack one ocular plate. One of them, E-1-57, also lacks a genital plate (adjacent to the missing ocular). The other, Y-1-563, has four good genital plates and possibly a fifth

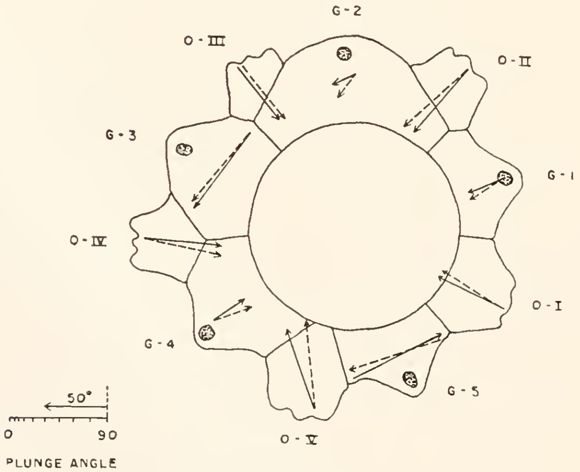

FIGURE 5. Average crystal orientations from Table III for six normal specimens (solid arrows) and six *situs inversus* specimens (dashed arrows). Morphology is generalized from Figure 1.

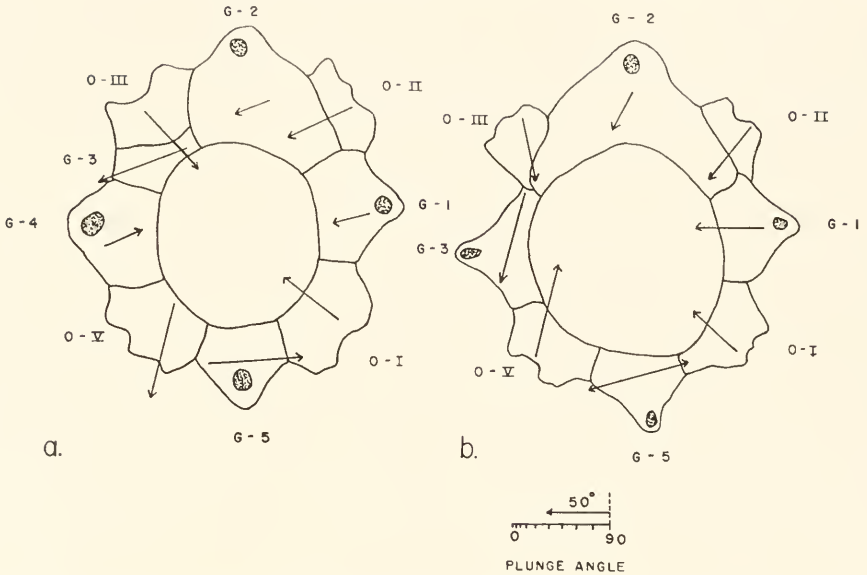

FIGURE 6. Crystal orientations in two tetramerous specimens of *Str. dröbachiensis*. *a*, #563. *b*, #57. (In #57, the arrow for plate G-5 has two points because of a 0° plunge.)

which, though lacking a genital pore, occupies a morphologically reasonable position. Apical systems of the two specimens are shown in Figure 6, and the numerical data on their crystal orientations are given at the bottom of Table II.

In both specimens, genital plates 3 and 5 are clearly recognizable from the crystal data. They have azimuths near 90° (plus or minus) and low plunges. The crystal orientations of the other genital plates are reasonable candidates for G-1, G-2, or G-4, although there is considerably more variation in orientation than is encountered in normal specimens. In specimen E-1-57, the plates identified as G-3 and G-5 have no intervening genital plate. Thus, the missing genital plate must be G-4.

In specimen Y-1-563, O-IV is evidently the missing ocular. This conclusion is based simply on the fact that there is no ocular plate between the plates that have been identified as G-3 and G-4. In specimen E-1-57, the missing ocular is either O-IV or O-V. The normal crystallography of these two plates is not sufficiently different to enable us to choose between the alternatives. The ocular standing between G-3 and G-5 is broadly insert, suggesting O-V. If this morphological interpretation is correct, the missing ocular is O-IV. Because the morphological development of plates is inevitably distorted to some degree by the tetramerous condition, the interpretation must be somewhat tentative.

DISCUSSION

It is clear from the data that the morphological inversion in *situs inversus* specimens is mimicked by a parallel crystallographic inversion. This places the time of inversion no later than the earliest stages of metamorphosis, when the echinus rudiment is formed in the pluteus larva (Raup, 1965). This strengthens the suggestion made by Swan (1966) that the inverted individuals are those individuals in which the echinus rudiment develops on the right side (instead of the left) of the pluteus. This would produce a mirror image inversion of both morphology and crystallography.

The origin of the tetramerous condition cannot be placed so exactly in time because it involves a deletion of parts. Since we are dealing only with adult specimens, the crystallographic data do not provide clues to when during development the deletion took place. The fact that the tetramerous specimens lack any trace of a fifth ambulacrum suggests, however, that the fifth ocular was missing in the echinus rudiment. This may mean that the postero-dorsal rod on the left side of the pluteus (which normally produces O-IV) was missing in the larval skeleton. The fact that on other bases Jackson (1912) considered his five perfectly tetramerous specimens of *S. droebachiensis* to have ocular IV and the associated ambulacrum missing and Kier and Grant (1965) considered the missing ocular and ambulacrum from their tetramerous specimens (one each) of *Meoma ventricosa* and *Encope michelini* to be the same makes one wonder if there may not be some relationship which makes this deletion more likely than others. For the tetramerous variants of other species examined by Jackson (1912, 1927), however, various other oculars and associated ambulacra were postulated as being the missing parts.

Quite apart from the subject of aberrant development, the data presented in this paper shed light on the general problem of the precision with which crystals are oriented in the echinoid apical system. It was suggested in an earlier paper (Raup, 1965) that "each species (or genus) has its own crystallographic 'signature' " (p. 938). This idea may be carried further with the present data.

Note in Table III that the average plunge angles for ocular plates of normal specimens vary from 31.2° to 46.7°. The comparable values for *situs inversus* specimens in Table III have about the same absolute range (27.6° to 41.5°) but each is about 5° lower than its normal counterpart. The important thing here is that the relative ranking of oculars (with respect to plunge) is essentially the same in the two groups. It is not enough to say that *c*-axes of oculars plunge at angles of about 40°. Rather, it appears that the plunge of O-III (or O-II in *situs inversus* specimens) is the highest; O-I and O-IV are the next highest; these are followed by O-II; and O-V has the lowest plunge. Similar patterns are evident in the other plunge and azimuth data in Table III.

The average values in Table III represent four species and collections from a wide geographic area. Inspection of Table II makes it possible to explore the crystallographic signature at the population level. For example, the first two specimens listed in Table II are from a single population at York, Maine. With a few exceptions, the two specimens are more similar to each other in crystal orientation pattern than either is to specimens from other populations and species.

The samples are much too small to make definitive statements on intraspecific or intrapopulation variation. The patterns suggest, however, that further study might be extremely fruitful as applied to general problems of echinoid variability. It is indeed possible that at the population level, crystal orientations may be influenced by local environment.

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STUDIES ON MEMBRANE TRANSPORT. I. A COMMON TRANSPORT SYSTEM FOR SUGARS AND AMINO ACIDS?[1]

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It has been reported that sugars, particularly galactose, interfere with the absorption of amino acids by intestinal tissues (Newey and Smyth, 1964; Saunders and Isselbacher, 1965; Alvarado, 1966; Annegers, 1966; Chez *et al.*, 1966), by microorganisms (Ames, 1964; Kepes, 1964), and by tapeworms (Kilejian, 1966; Read *et al.*, unpublished data). Various suggestions have been made concerning the mode of action of sugars, including competition for energy sources (Newey and Smyth, 1964), formation of a toxic metabolite (Saunders and Isselbacher, 1965), and direct allosteric effects in a polyfunctional carrier system. Kilejian (1966), working in the present author's laboratory, showed clearly that previously absorbed glucose inhibits the subsequent absorption of proline by the rat tapeworm, *Hymenolepis diminuta.* None of the suggested mechanisms for sugar inhibition of amino acid transport seem to be consistent with Kilejian's findings.

The present paper is concerned with interactions of sugars and amino acids in their absorption by intestinal mucosa of the smooth dogfish, *Mustelus canis.* Preparations of the spiral intestine from elasmobranchs have advantageous qualities for experiments on tissue uptake of metabolites; both sides of the flat excised valve are constituted of mucosal cells and the reproducibility of multiple samples is satisfactory (Read *et al.,* 1960).

MATERIALS AND METHODS

Smooth dogfish were used within 1 to 5 days after capture in the waters off Woods Hole, Massachusetts. Animals were killed by blows on the head and the spiral intestine rapidly removed. The third or fourth valve was excised and spread flat on a chilled plate. Replicate samples about 1 cm.^{2} (60 to 80 mg. of wet tissue) were cut with sharp scissors and removed to chilled elasmobranch saline containing 300 mM urea (Read *et al.*, 1960). As many as 36 samples were readily obtained from a single valve and were used immediately in experiments. Incubations of single samples were carried out at 20° C. in 8- or 10-ml. volumes of media. Preliminary experiments showed that gassing with 95% O_2 – 5% CO_2 or 95% air – 5% CO_2, with the addition of bicarbonate buffer, did not increase the rate of amino acid transport. Hence, the incubations were performed in a shaker bath

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without gassing. The elasmobranch saline contained 25 m*M* tris-maleate buffer (pH 7.4). Assays of cycloleucine-^{14}C (1-aminocyclopentane-1-carboxylic acid) and galactose-^{14}C were made on dried aliquots of 70% ethanol extracts of tissue samples. Wet weights of tissue were obtained by blotting samples on hard filter paper and weighing rapidly on a torsion balance. Dry weights of tissues were obtained after heating samples at 95° C. for 24 hours.

Labeled compounds were obtained from New England Nuclear Corporation; the L-amino acids and sugars used were obtained from the California Corporation for Biochemical Research.

RESULTS

The amino acid cycloleucine (1-aminocyclopentane-1-carboxylic acid), which is not metabolized by mammals (Christensen and Jones, 1962), was chosen for use in the present study after preliminary experiments were carried out to determine that the compound is transported and is not metabolized by dogfish intestinal tissues.

After 40-minute incubations of spiral valve tissue in 1.0 m*M* cycloleucine-^{14}C with continual gassing with 95% air-5% CO_2, the tissues were extracted with warm 70% ethanol. The residue was extracted five times with cold 70% ethanol, hydrolyzed, and tested for radioactivity; none was present. To the combined ethanol extracts from each sample, an equal volume of 0.2 N HCL was added and four volumes of chloroform. The material was partitioned three times for four hours in a rocking extractor. Aliquots of the aqueous phase were analyzed in the Technicon amino acid analyzer with an attached Packard scintillation flow cell system. The radioactivity in the samples was present only in cycloleucine. The chloroform extracts contained no radioactivity, and it was concluded that cycloleucine was not significantly metabolized by this tissue in this time period.

Since the movement of solute into mucosal cells was to be measured, a number of experiments were carried out to ascertain whether significant net movements of water occurred in various incubation media. These data are presented in Table I.

TABLE I

The effect of 10-minute incubations in several media on water content of Mustelus gut tissue. In salines with Na = 100 and Na = 25, Tris-Cl was substituted for deleted NaCl. Each value is mean of 10 determinations

Incubation medium	$\frac{\text{Dry wt.}}{\text{Wet wt.}} \times 100$	$\frac{\text{EtOH-extracted dry wt.}}{\text{Wet wt.}} \times 100$
Saline	23.1 ± 0.67	—
Saline	—	17.8 ± 9.77
Saline + 5 m*M* galactose	23.0 ± 0.58	—
Saline + 5 m*M* galactose	—	17.6 ± 0.64
Saline (Na = 100)	23.2 ± 0.62	—
Saline (Na = 100)	—	17.8 ± 0.74
Saline (Na = 25)	23.1 ± 0.71	—
Saline (Na = 25)	—	17.5 ± 0.36
Unincubated	23.7 ± 0.23	—
Unincubated	—	17.7 ± 0.74

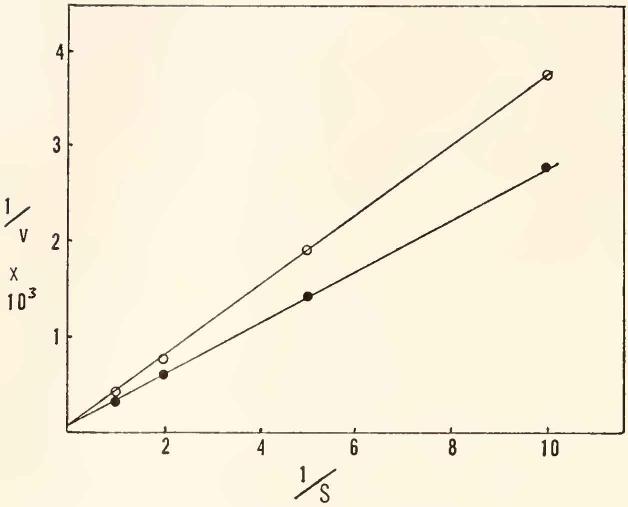

FIGURE 1. Uptake of cycloleucine with and without galactose. Spiral valve samples from one fish incubated for 10 minutes in media containing cycloleucine-^{14}C with 5 m*M* galactose (open circles) or without galactose (closed circles). S = m*M* cycloleucine, V = mμmoles/gram/10 minutes. Each point is mean of four replicates.

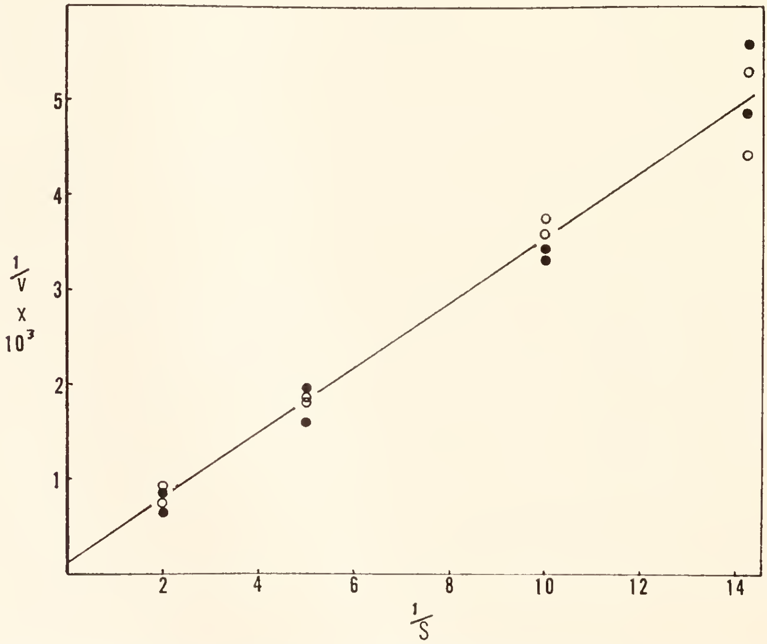

FIGURE 2. Uptake of cycloleucine with and without 5 m*M* galactose. Spiral valve samples incubated for 2 minutes. Other conditions as in Figure 1.

Water movements were considered to be of negligible significance in the subsequent experiments.

When mucosal tissues were incubated for 10 minutes with various concentrations of cycloleucine-^{14}C in the presence or absence of a constant concentration of galactose, cycloleucine uptake was inhibited by the sugar. In a Lineweaver-Burk plot, the inhibition appeared to be competitive in character (Fig. 1). However, when the incubation time was reduced to 2 minutes, the sugar produced no significant inhibition of cycloleucine uptake (Fig. 2). Previous studies had shown that the competitive inhibition of uptake of one amino acid by another is readily demonstrated in this tissue (Read *et al.*, 1960) and, in the present study, leucine was found to competitively inhibit cycloleucine uptake in 2-minute incubations (Fig. 3). When the tissue was preincubated for 10 minutes in galactose or certain other sugars, rinsed, and incubated for 2 minutes with cycloleucine-^{14}C without sugar, the uptake of cycloleucine was inhibited (Table II). Mannitol and sorbose, which are not transported by vertebrate intestinal tissue (Crane, 1960), were without effect on the subsequent uptake of cycloleucine. On the other hand, tissues incubated with 3-O-methyl glucose, α-methyl glucoside, glucose, or galactose, all of which are

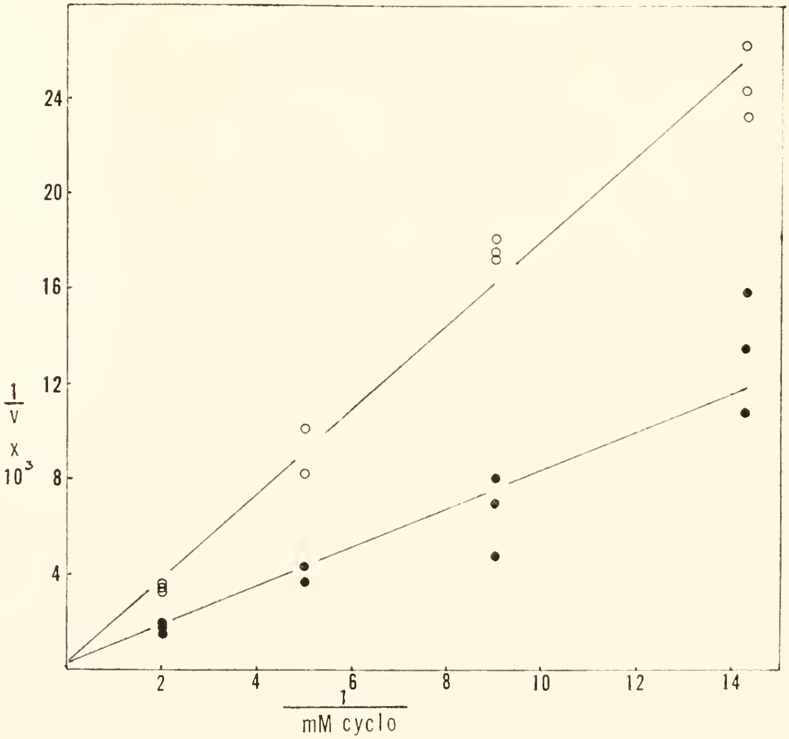

FIGURE 3. Inhibition of cycloleucine uptake by leucine. Spiral valve samples incubated for 2 minutes in media containing cycloleucine-^{14}C with 5 m*M* L-leucine (open circles) or without leucine. V = mμmoles/gram/2 minutes. Each point is an individual determination.

Table II

The effects of preincubation in various sugars on the subsequent uptake of cycloleucine-^{14}C by Mustelus intestinal tissue. Components of preincubation and incubation media added to buffered elasmobranch saline. Each value is mean of four determinations

Expt.	10-minute preincubation	2-minute incubation	Cycloleucine-^{14}C uptake (mμmoles/g./2 min.)
I.	5 m*M* mannitol	0.1 m*M* cycloleucine-^{14}C	225 ± 23.1
	5 m*M* mannitol	0.1 m*M* cycloleucine-^{14}C + 5.0 m*M* galactose	208 ± 19.2
	5 m*M* galactose	0.1 m*M* cycloleucine-^{14}C	137 ± 11.4
II.	5 m*M* mannitol	0.1 m*M* cycloleucine-^{14}C	240 ± 12.4
	5 m*M* mannitol	0.1 m*M* cycloleucine-^{14}C + 5 m*M* galactose	243 ± 16.8
	5 m*M* glucose	0.1 m*M* cycloleucine-^{14}C	192 ± 12.3
	5 m*M* glucose	0.1 m*M* cycloleucine-^{14}C + 5 m*M* glucose	178 ± 8.1
III.	5 m*M* mannitol	0.1 mM cycloleucine-^{14}C	226 ± 12.4
	5 m*M* sorbose	0.1 m*M* cycloleucine-^{14}C	220 ± 14.2
	5 m*M* alpha-methylglucoside	0.1 m*M* cycloleucine-^{14}C	178 ± 7.9
	5 m*M* 3-O-methylglucose	0.1 m*M* cycloleucine-^{14}C	164 ± 7.7
	5 m*M* galactose	0.1 m*M* cycloleucine-^{14}C	151 ± 6.7

actively transported by intestinal mucosa, showed a decreased uptake of cycloleucine when subsequently incubated with the amino acid. Addition of the glycoside phlorizin to the galactose-containing preincubation medium prevented the inhibition of cycloleucine uptake. Phlorizin itself was without effect on cycloleucine absorption (Table III). Other experiments showed that the addition of phlorizin after preincubation with galactose did not reverse the inhibition of cycloleucine uptake.

When tissue samples were preincubated for 10 minutes in 5 m*M* galactose, followed by 2-minute incubation in various concentrations of cycloleucine, a Lineweaver-Burk plot of the data suggested that the inhibition is competitive in character (Fig. 4). At any rate, K_m is changed without a change in V_{max}.

Table III

The effect of phlorizin in blocking the galactose inhibition of cycloleucine-^{14}C uptake. Preparations incubated as in Table II. Each value is mean of four replicate determinations

10-Minute preincubation	2-minute incubation	Cycloleucine-^{14}C uptake (mμmoles/g./2 min.)
Saline	0.1 m*M* cycloleucine-^{14}C	230 ± 10.8
5 m*M* galactose	0.1 m*M* cycloleucine-^{14}C	146 ± 8.6
5 m*M* galactose + 0.5 m*M* phlorizin	0.1 m*M* cycloleucine-^{14}C	215 ± 12.2
0.5 m*M* phlorizin	0.1 m*M* cycloleucine-^{14}C	228 ± 12.1
Saline	0.1 m*M* cycloleucine-^{14}C + 0.5 m*M* phlorizin	233 ± 11.7

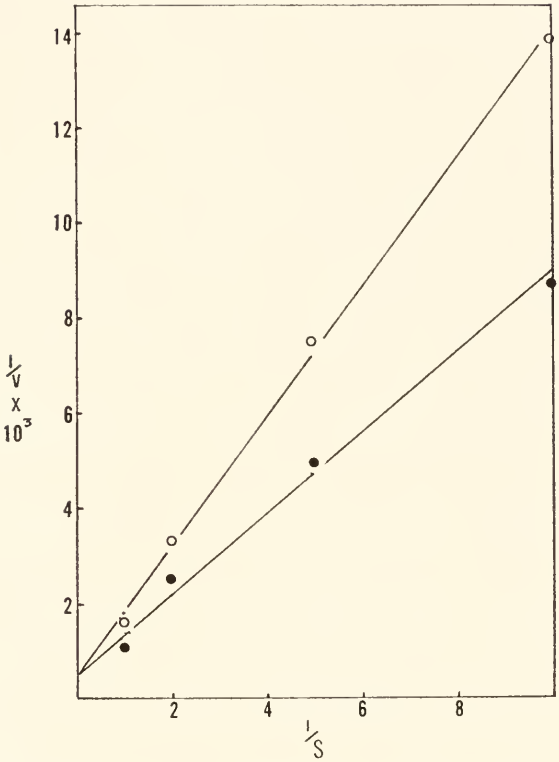

FIGURE 4. Inhibition of cycloleucine uptake by preincubation of tissue with galactose. Tissue samples were preincubated for 10 minutes in elasmobranch saline with 5 m*M* galactose or without galactose. The uptake of cycloleucine-^{14}C during a 2-minute incubation without galactose in the medium was then determined. Open circles denote 2-minute uptake of samples preincubated with galactose; closed circles are 2-minute cycloleucine uptake of samples preincubated in saline without galactose. S = m*M* cycloleucine; V = mμmoles/g./2 minutes. Each point is mean of four replicates.

When tissues were incubated for various time intervals in 5m*M* galactose, followed by a 2-minute incubation in cycloleucine, the inhibition of amino acid uptake was a function of the time of previous exposure to the sugar up to 10 minutes (Fig. 5). Since this was consistent with the view that the inhibition was a function of the amount of sugar previously absorbed by the tissue, it was reasoned that varying the concentration of galactose in a fixed incubation time should produce varying degrees of inhibition of cycloleucine uptake. Such is indeed the case. Preincubation of tissue for 10 minutes in concentrations of galactose ranging from 0.5 to 24 m*M* produced inhibitions of varying intensity up to a maximum attained at about 4 m*M* (Fig. 6). Such an attainment of a maximum would be expected in a short fixed time period if the inhibition is dependent on the amount of sugar absorbed and if sugar absorption follows saturation kinetics.

At this point the data seemed to indicate that absorption of galactose affected subsequent uptake of an amino acid but did not allow any conclusion as to whether energy-requiring mechanisms might be involved. Preliminary experiments showed that treating the tissue with 2,4-dinitrophenol or subjecting it to complete anoxia resulted in a failure of galactose accumulation, the sugar in the tissue coming to concentration equilibrium with that in the external medium. Experiments were carried out to determine whether galactose inhibited amino acid uptake in such preparations. As with untreated tissues, galactose produced no significant inhibition of cycloleucine uptake in 2-minute incubations, but in 10-minute incubations galactose inhibited cycloleucine uptake. Similar results were obtained with tissues incubated under nitrogen. As will be seen in Table IV, when sodium in the incubation medium was markedly reduced, the galactose effect was not observed with dinitrophenol-treated tissues. This is consistent with the idea that sodium is required for sugar absorption, as has been demonstrated with a number of other tissues (Crane, 1965).

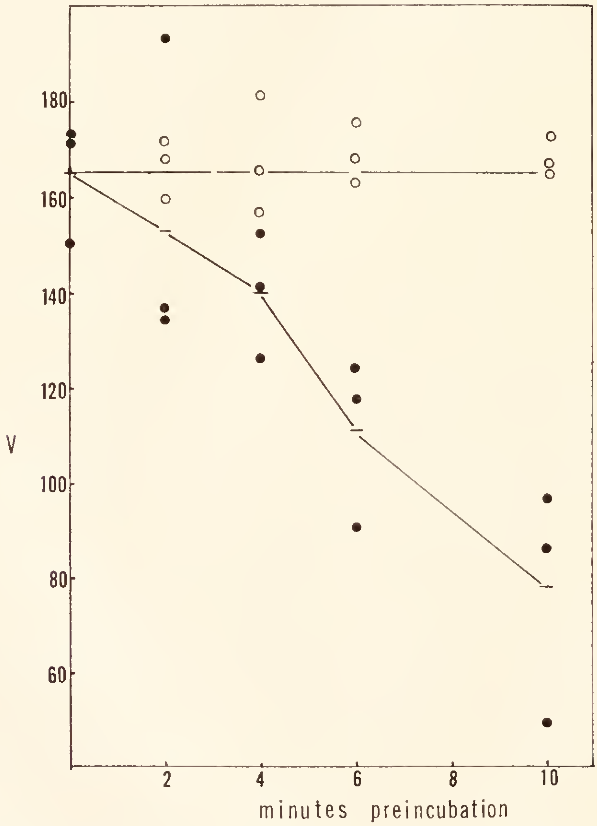

FIGURE 5. Effect of duration of preincubation with galactose on subsequent uptake of cycloleucine. Samples were preincubated for time intervals shown with 5 mM galactose (closed circles) or without galactose (open circles). The uptake of cycloleucine-^{14}C was then determined in a 2-minute incubation in galactose-free medium. Cycloleucine at 0.1 mM. V = mμmoles/g./2 minutes. Each point is an individual determination.

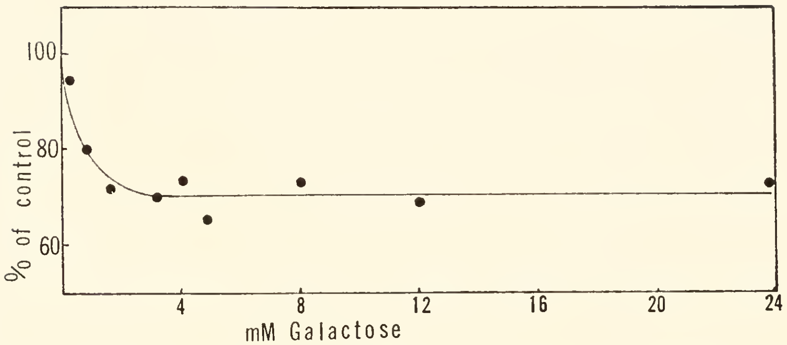

FIGURE 6. Effect of concentration of galactose in preincubation medium on subsequent uptake of cycloleucine. Samples incubated in galactose at indicated concentrations followed by 2-minute incubation in galactose-free medium containing 0.1 mM cycloleucine-^{14}C. Each point is mean of triplicates.

The effect of sodium and potassium on galactose uptake by dogfish intestine was examined and it was found that sodium is indeed required in the transport of galactose while potassium produces an inhibition of galactose uptake when sodium is absent from the incubation medium (Fig. 7). The possible interaction of sodium and potassium in the galactose transport system was examined. Potassium appears to antagonize sodium activation of galactose transport (Fig. 8).

Since sodium might also activate the amino acid transport system and this might have bearing on the relationship between sugar transport and amino acid transport, the effect of sodium on cycloleucine uptake was examined. It was found that cycloleucine transport is sodium-dependent (Fig. 9). However, unlike the sugar system, potassium does not appear to antagonize the sodium activation of cycloleucine transport (Fig. 10).

TABLE IV

All samples were preincubated for 10 minutes in elasmobranch saline containing 0.05 mM 2,4-dinitrophenol and 0.2 mM cycloleucine-^{14}C. They were then transferred to media containing the same concentrations of dinitrophenol and cycloleucine-^{14}C plus the additions shown below. Tris-Cl was substituted for sodium deleted from the incubation medium. Each value is mean for four replicates

10-min. incubation	Final tissue conc. (mμmoles/g.)
Na = 10 mM Galactose = 2 mM	631 ± 30.4
Na = 10 mM	656 ± 28.6
Na = 225 mM Galactose = 2 mM	821 ± 31.3
Na = 225 mM	1,003 ± 40.6
Uptake during preincubation = 549 ± 27.8	

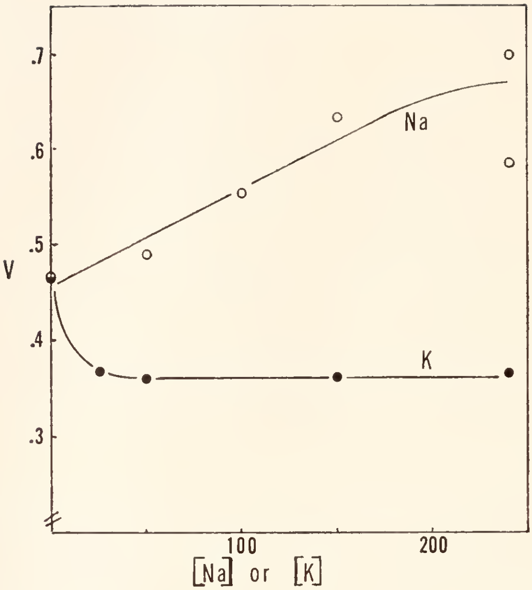

FIGURE 7. The effect of sodium or potassium on the uptake of galactose-^{14}C. Tris-Cl was substituted for sodium chloride or potassium chloride in media tested. Magnesium and calcium salts were held at concentrations for elasmobranch saline. Galactose was added at a concentration of 0.5 m*M* and uptake in 2-minute incubations determined. Sodium or potassium concentration is m*M*; V = μmoles/g./2 minutes. Each point is mean of four replicates.

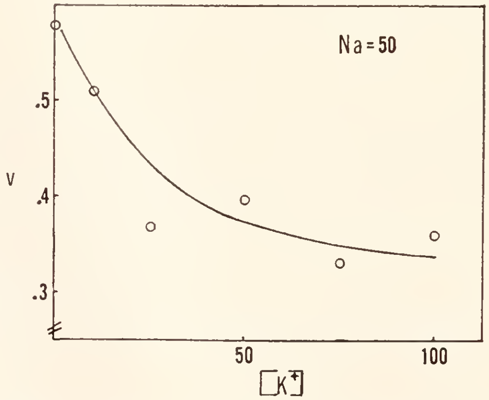

FIGURE 8. The potassium antagonism of sodium activation of 2-minute galactose uptake. Sodium concentration was held constant and potassium concentrations were varied. Tris-Cl was substituted for the normal sum of KCl and NaCl deleted from elasmobranch saline. Concentrations are m*M*. Galactose-^{14}C was present at 1.0 m*M* and V = μmoles/gram/2 minutes. Each point is mean of four replicates.

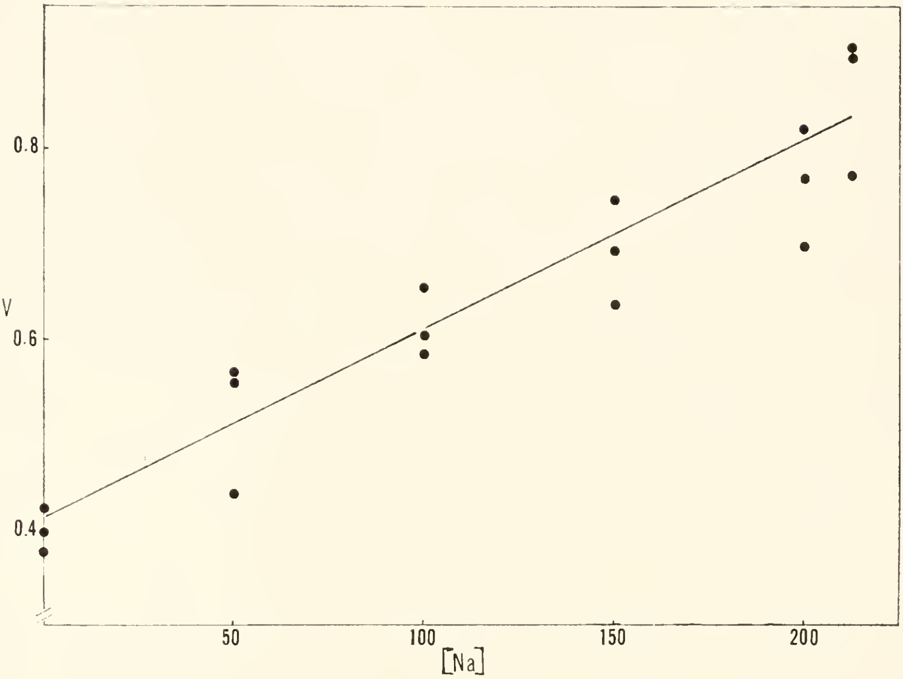

FIGURE 9. Activation of cycloleucine transport by sodium. Samples were incubated for 5 minutes in elasmobranch saline in which NaCl was varied with Tris-Cl added to substitute for deleted amounts. Cycloleucine-^{14}C was 0.2 m*M* in all vessels. Sodium concentration is m*M* and V = μmoles/gram/5 minutes. Each point is an individual determination.

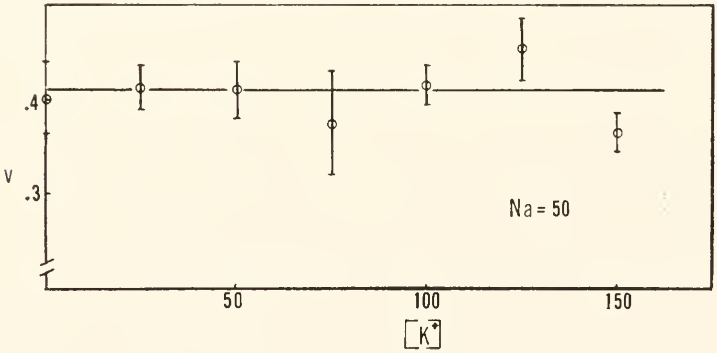

FIGURE 10. The effect of potassium on 5-minute cycloleucine uptake in sodium-deficient media. Sodium concentration was held at 50 m*M* and potassium concentrations were varied. Tris-Cl was substituted for the normal sum of KCl and NaCl deleted from elasmobranch saline. Salt concentrations are m*M*. Cycloleucine concentration was 0.2 m*M* in all vessels and V = μmoles/gram/5 minutes. Each point is mean of three samples.

Discussion

The data now available allow reconsideration of some previously suggested mechanisms by which galactose might inhibit amino acid uptake. The formation of an inhibitory metabolite, galactose-1-phosphate, suggested by Saunders and Isselbacher (1965), seems quite improbable since glucose, 3-O-methyl glucose, and α-methylglucoside also act as inhibitors. Previous accumulation of either glucose or galactose also affects subsequent absorption of proline or cycloleucine by the rat tapeworm *Hymenolepis diminuta* (Kilejian, 1966; Read, unpublished), and Read *et al.* (in preparation) found that glucose markedly decreased the steady state level of valine accumulated by the tapeworm *Calliobothrium.*

Although the number of sugars tested in the present study is not exhaustive, the results suggest that inhibition of amino acid uptake is only produced by sugars which are transported by the intestine. This might be expected in view of the strong evidence that the inhibitory sugars exert their effect after or during sugar absorption and do not appear to inhibit amino acid uptake by competition for a common transport mechanism. In contrast, the experiments with leucine indicate that this amino acid inhibits cycloleucine uptake by competition for a transport mechanism and that the competition occurs at the external interface of the cell. The blocking of the galactose inhibition of amino acid uptake by the sugar transport inhibitor phlorizin is also consistent with the interpretation that sugar absorption is necessary for inhibition of cycloleucine uptake. Alvarado's (1966) hypothesis that there is a common membrane carrier for sugars and amino acids in the mammalian mucosal cell was based on results obtained in incubations of 10-minute duration. In incubations of similar duration, we also obtained inhibitions quite comparable to those reported by Alvarado, whereas in 2-minute incubations, or in 10-minute incubations to which galactose was added during the last 2 minutes, no significant inhibition by the sugar was produced. It seems likely that in Alvarado's experiments the galactose entering in the early portion of the incubation period produced inhibition of amino acid uptake during the latter part of the incubation period. Alvarado's hypothesis of a single polyfunctional membrane carrier for sugars and amino acids does not seem to be a tenable one, at least for the dogfish intestine.

Although both galactose and cycloleucine transport systems are activated by sodium, the difference in the effects of potassium on the two systems is significant. Potassium antagonizes the sodium activation of the galactose transport system but does not antagonize sodium activation of cycloleucine transport. This also argues for the separateness of the transport systems for sugars and amino acids and implies that sodium ions activate the two systems independently. The sodium activation of amino acid and sugar transport systems resembles that seen in a variety of other tissues and organisms (Crane, 1965). However, the sodium requirement for cycloleucine uptake in dogfish intestine appears to differ from that of pigeon red cells, in which Vidaver (1964) reported that two sodium ions are required to activate the membrane carrier for glycine. Vidaver also found that potassium did not antagonize sodium activation of glycine transport in pigeon red cells.

Although the hypothesis of a common carrier is not acceptable, the data of the present paper, as well as those of Alvarado (1966) and Chez *et al.* (1966), may be consistent with the conclusion that the amino acid and sugar transport systems are in close proximity in the cell membrane.

Competition between galactose and the amino acid for energy sources has been ruled out by demonstration that previously absorbed galactose inhibits amino acid uptake in preparations poisoned by 2,4-dinitrophenol or by anoxia. This has further shown that accumulation of sugar, an energy-requiring phenomenon, is not directly involved in the inhibition. It may be emphasized that there is no evidence available to show that the transport event in sugar or amino acid absorption is itself energy-requiring. Accumulation of these compounds must require energy but there is no evidence that energy of metabolic origin is involved in the initial events of membrane transport. As a matter of fact, the only difference between active transport and facilitated diffusion is the accumulation of a substance against a concentration difference and the energy requirement may involve an independent event.

It is suggested that the galactose inhibition of cycloleucine uptake is related to the sodium activation of both systems and that galactose uptake produces an increase in the intracellular sodium in that region of the cell involved in amino acid and sugar absorption. This localized increase in sodium would enhance the probability for efflux of amino acid from the cell and lower the net transport. This hypothesis may be amenable to experimental test.

Summary

1. The uptake of cycloleucine (1-aminocyclopentane-1-carboxylic acid) by dogfish intestinal tissue is inhibited by galactose in 10-minute incubations but not in 2-minute incubations.

2. Preincubation of the tissue in galactose inhibits subsequent uptake of cycloleucine in 2-minute incubations without sugar. Addition of phlorizin to the preincubation with galactose abolishes inhibition. The inhibition by galactose, and other actively transported sugars, is effected during or after absorption of the sugar and is not a competition for a common site at the external interface. Leucine, on the other hand, competitively inhibits cycloleucine uptake in 2-minute incubations.

3. The inhibitory effects of galactose are dependent on time of preincubation and concentration of the sugar.

4. In 2,4-dinitrophenol-inhibited tissues, the previous absorption of galactose inhibits the subsequent uptake of cycloleucine.

5. Both galactose uptake and cycloleucine uptake are sodium-dependent. However, while potassium antagonizes sodium-activated sugar uptake, it is without effect on amino acid uptake.

6. The data are discussed and several hypotheses for mechanisms of sugar inhibitions of amino acid absorption are rejected. An hypothesis is offered that sugar produces the inhibitory effect on amino acid absorption by highly localized alteration of intracellular sodium concentration.

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OSMOTIC REGULATION AND ADAPTIVE REDUCTION OF WATER-PERMEABILITY IN A BRACKISH-WATER CRAB, RHITHROPANOPEUS HARRISI (BRACHYURA, XANTHIDAE)

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Recent studies by various authors have made it evident that among crustaceans of brackish and fresh waters there exists considerable diversity in the combinations of mechanisms of primary importance in osmotic regulation. This is to be expected because the crustaceans of such waters do not represent a single evolutionary line, but rather are representative of different orders and families, some of whose species have independently adapted to conditions of low salinity. While it is probable that certain physiological mechanisms of adaptation to brackish or fresh water are shared by different crustacean groups, there is no *a priori* reason to assume that all groups emphasize a common set of mechanisms. This has been especially clearly indicated in the work of Shaw (1959, 1961b), who has shown that the adaptation of the African crab *Potamon niloticus* to fresh water takes the form of a lowering of body permeability to both salts and water, with the production of a very small amount of blood-isotonic urine. In contrast, fresh-water crayfishes show a lowering of surface permeability to salts, but not so much so to water; the urine is consequently copious, but salts are actively recovered from the urine, which is rendered hypotonic to the blood. That there is no necessary relationship between a fresh-water habitat and the production of hypotonic urine has been indicated by Lockwood (1961), who has shown hypotonic urine production in both fresh-water and brackish-water amphipods, and by Parry (1957), who has demonstrated that the fresh-water prawn *Palaemonetes antennarius* produces a copious but blood-isotonic urine.

The true crabs (Brachyura) are a basically marine group, from several families of which representative genera and species have independently made physiological adaptations to life in fresh or nearly fresh waters. It would be of interest to know whether or not the extremely low urine production of *Potamon* (Shaw, 1959) represents the culmination of an adaptive trend toward reduction of water-permeability common to, and expressable as a generalization for, those brachyurans entering fresh waters. On the other hand, as pointed out by Potts and Parry (1964, p. 174), the apparent low water-permeability of *Potamon* may represent either a uniquely low water-permeability ($\frac{1}{10}$ that of any other fresh-water crustacean) or it may be the result of extra-renal water excretion. Either of the latter two possibilities would be inconsistent with the generalization that reduced permeability to water is a mechanism utilized by euryhaline or fresh-water Brachyura as a group. The crabs studied by Shaw include *Carcinus* (Portunidae), *Eriocheir* (Grapsidae) and *Potamon* (Potamonidae). Although these are arranged in a series of increasing adaptedness to low salinity and fresh water, they do not constitute an actual evolutionary series related by descent within the Brachyura. Rather, *Eriocheir* is in the grapsoid division of the Brachyrhyncha while *Carcinus* and *Potamon* are in the cancroid division of that group, and hence are more closely related to each other than either is to *Eriocheir*. In order better to assess the possibile generalization that a reduction in water-permeability is a physiological mechanism common to the cancroid crabs represented by *Carcinus* and *Potamon* it is of interest to evaluate the osmotic performance of a cancroid crab which, in its euryhalinity, stands between *Carcinus* and *Potamon*. This paper reports on the small crab *Rhithropanopeus harrisi* (Gould), which appears to be the best-adapted to low salinities of any of the Xanthidae, a large family of marine and brackish-water crabs.

Ecology and Distribution

Rhithropanopeus harrisi has its center of distribution on the central Atlantic coast of the United States. From there it is thought to have been introduced into the formerly brackish Zuider Zee of Holland (Buitendijk and Holthuis, 1949) some time before 1874. Since 1936 it has been recorded from Germany, Denmark, and southern Russia. In Holland, the form was known prior to 1949 as *Heteropanope tridentata* (Maitland), formerly *Pilumnus*, and is given subspecific rank as *Rhithropanopeus harrisi* (Gould) *tridentatus* (Maitland) by Buitendijk and Holthuis (1949). *R. harrisi* has also reached the west coast of the United States, where it has become established in estuarine waters of low salinity about the San Francisco Bay estuarine system (Jones, 1941). In this region, as elsewhere, adults may also be found occasionally in fresh water, but the species does not appear to be capable of reproducing therein.

Previous studies by Jones (1941) and Kinne and Rotthauwe (1952) have shown that *Rhithropanopeus harrisi* hyper-regulates in lower salinities (below *ca.* 60% sea water) and conforms or remains slightly hypertonic to higher salinities. Nothing is known of the mechanisms involved; hence the following observations on water-permeability and urine production represent a first assessment of osmoregulatory mechanisms in the species. Crabs for this study were collected in the Napa River, an estuary opening into San Francisco Bay, where they occur in numbers under rocks laid as a protective layer on muddy intertidal banks. Salinities are variable, being less than 5% of sea water in the rainy winter season, but above 25% sea water in summer.

As an experimental animal for comparative physiology, *Rhithropanopeus harrisi* is well suited by virtue of its wide salinity tolerance, abundance, and its extensive (and increasing) geographical distribution, but for some purposes its small size, up to 2 cm. across the carapace and rarely exceeding 4 grams in weight, at least in this area, is a disadvantage. In contrast, *Eriocheir* and *Carcinus* commonly exceed 100–150 grams, and *Potamon* 20–30 grams.

Methods

Crabs undergoing experimental adaptations were maintained at 13–14° C. and were fed chopped fish twice weekly. Prior to determination of "adapted" values for blood and urine, crabs were held at the test salinity at least 5 days; experience has shown that osmoregulatory response is esssentially complete within 2 days in the steps of not over 25% sea water used in experimental changes of salinity.

D_2O determinations in blood samples were by the simplified bromobenzene-kerosene gradient column method (Smith, 1964) using D_2O standards made up in distilled water. Each blood sample was drawn in a sharp capillary by puncture of the arthrodial membrane at a leg-base, and introduced into an "Aloe" disposable pipette (short style) that had been boiled in distilled water and oven-dried. A light plug of cotton was inserted past the constriction, the sample drop placed between the mouth and the constriction, the pipette tightly closed at the base by a small cork, and the tip sealed in a flame. The sealed pipettes were laid on a slide-warmer set at 50° C., with their tips either in air or resting on a chilled brass block, and the samples distilled to dryness overnight. D_2O concentrations could be estimated in the gradient columns to about 0.1% (mean of 2), and were expressed as a percentage of the concentration of D_2O in the bathing media.

Chloride determinations of blood, urine, and media were made with a Buchler-Cotlove chloridometer. Samples were collected in 1- or 2-mm.^{3} disposable pipettes (Drummond "microcaps"). For collection of urine, crabs were dried with absorbent paper, and the area around the urinary pores coated with beeswax applied with a warm cautery needle, so that emitted urine did not drain away into the sutures next to the apertures. The opercular plate covering the antennal gland aperture was lifted by a fine hooked needle. The crab in response might or might not emit a drop of clear urine, some of which was collected by capillarity in a microcap. Attempts to catheterize these small crabs (mostly less than 2 grams) have been unsuccessful or resulted in bleeding.

Other methods are described in connection with certain experiments, below.

Experimental

A. *Blood concentration as a function of salinity*

In Figure 1 are plotted data showing the pattern of chloride regulation in the blood of *Rh. harrisi* after adaptation to selected salinities. Also in Figure 1 are plotted the means of a series of determinations of the osmotic pressure of the blood obtained by a student, Miss Etta Kwan, using the comparative melting point method of Gross (1954) with reference to NaCl standards. Since the osmotic pressure of the blood is not entirely the result of its Na^+ and Cl^- concentrations, the osmotic pressure curve lies, as expected, a little above that for chloride. In general, the results of Jones (1941) and Kinne and Rotthauwe (1952) are confirmed, except that our data show a slight hypotonicity of blood of crabs in salinities above *ca.* 70% sea water, whereas the previous authors' figures indicate a slight hypertonicity in that upper salinity range. From about 60% sea water down to nearly fresh water there is a clear state of hyper-osmotic and chloride regulation, and in this range of salinities we may expect to find regulatory mechanisms most active. The level of chloride regulation at low salinities (but above fresh water, in which most crabs become sluggish, and in which a high percentage die after a few days) approximates the value of 242 mM/L. reported by Shaw (1959) for *Potamon niloticus* in fresh water. *Rhithropanopeus* thus behaves as do other Brachyura, maintaining a high

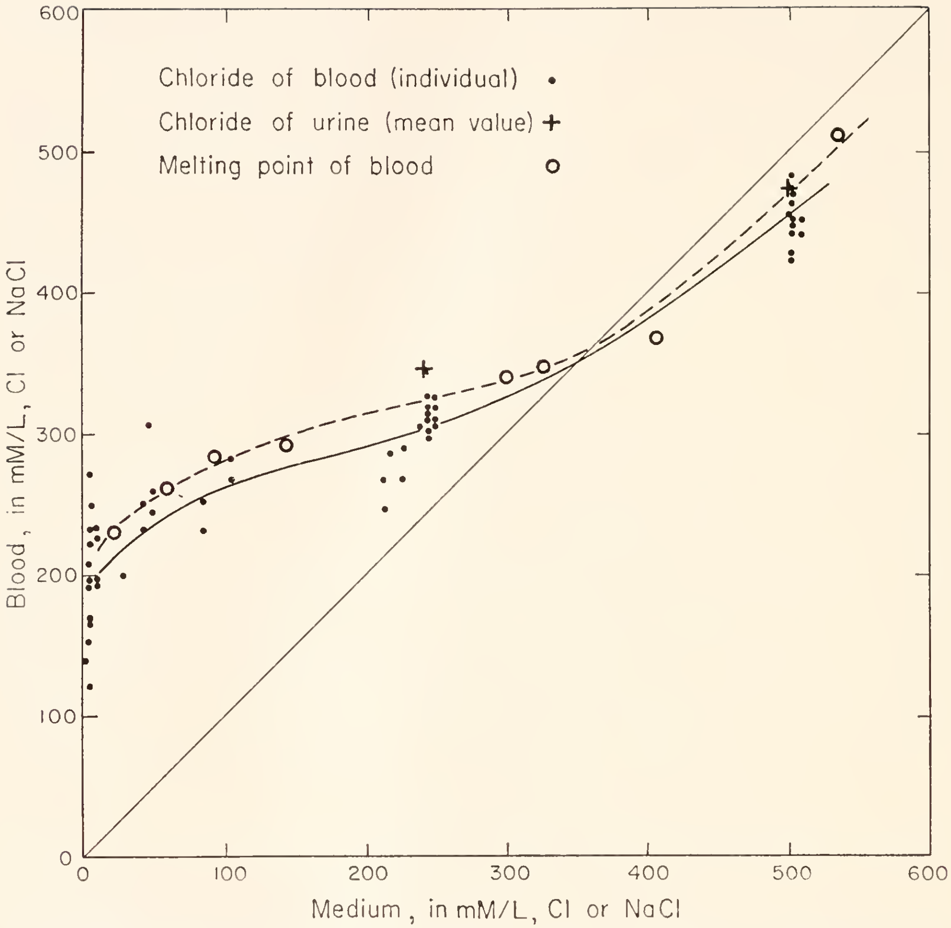

FIGURE 1. Concentration of blood and urine as a function of salinity (expressed in mM of Cl^- or NaCl per liter). Circles: melting point of blood expressed as equivalent NaCl concentration, each circle the mean of 8–16 determination on different animals. Dots: Cl^- from duplicate samples on individual crabs. Crosses: mean values for urinary Cl^-, based on 10 of animals furnishing blood values at salinities indicated. Melting point, urinary Cl^-, and 13 of blood Cl^- data obtained by Miss Etta Kwan.

blood concentration, rather than reducing osmotic stress by lowering blood concentration to the degree shown by crayfishes.

B. *The chloride concentration of the urine*

Urine in *Rh. harrisi* is fairly copious. The collection of urine was carried out under a dissecting microscope at 18×, so that any admixture of blood could be readily detected by the observation of blood cells in the sample; any such samples were discarded. Following the obtaining of two urine samples, blood samples were obtained in duplicate by puncture of the arthrodial membrane at a leg base and

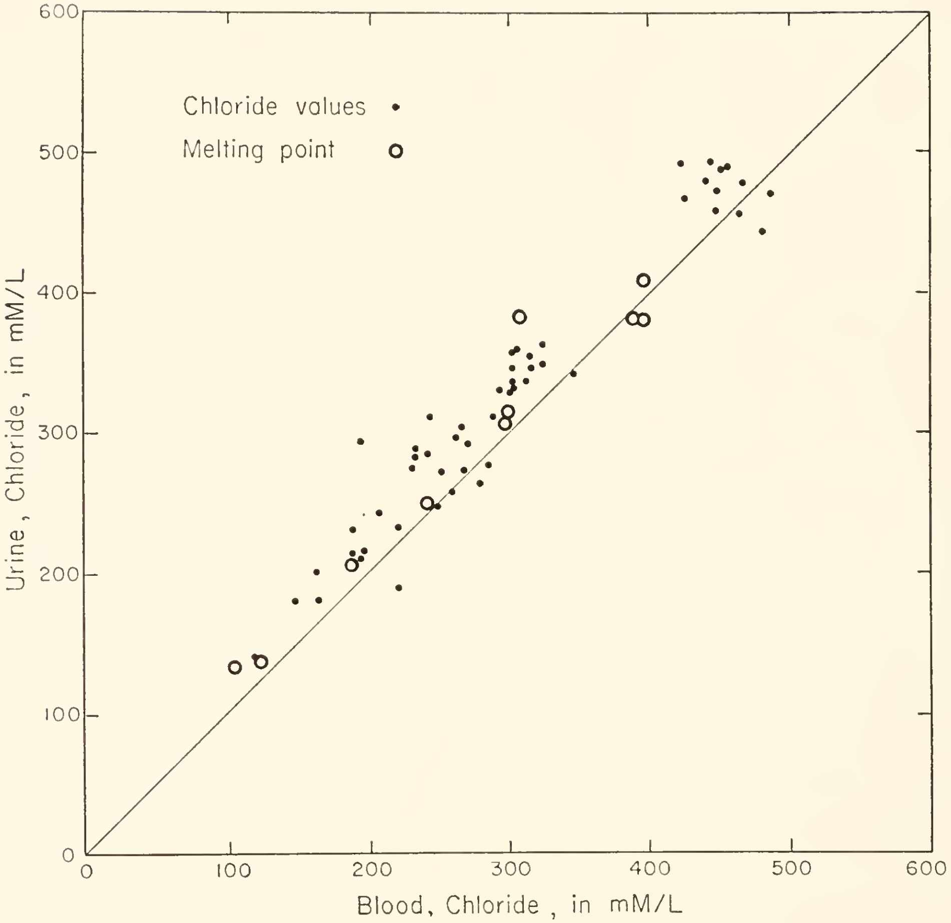

FIGURE 2. Urinary Cl⁻ and osmotic pressure as function of Cl⁻ and O.P. of blood. Each dot based on duplicate blood and urinary Cl⁻ determinations on same animal. Each circle based on freezing point data, expressed as equivalent NaCl, obtained by Miss Etta Kwan and used also in Figure 1.

insertion of a disposable pipette. The results of such paired determinations are shown in Figure 2, in which the values for urine samples (means of two) are plotted against the mean values of the duplicate blood samples. It is clear that the chloride of the urine is slightly greater than that of whole blood. However, since the blood contains proteins, and hence has a lesser water-content than the urine, it is presumed that urine and blood, on water-content basis, are isotonic in respect to chloride. Robertson (1949) has calculated a mean water content of 93.5% by weight in the blood of 3 marine crustacean species. Such a value would essentially account for the difference in chloride concentration between whole blood and urine in *Rh. harrisi,* and in part for the apparent hypotonicity of the blood of animals in sea water. However, since both chloride and osmotic pressure of the urine are slightly below those of sea water when animals are adapted to that medium, it seems probable that *Rh. harrisi* shows a slight ability to hypo-regulate in sea water, as is common among brackish-water decapod crustaceans. *Rh. harrisi* resembles all other species of crabs so far studied in not having adopted the production of hypotonic urine as an osmoregulatory device. The apparently copious amount of urine suggested a high water intake, and attempts were made to evaluate urinary output and water intake as functions of salinity.

C. *Urine volume and rate of production*

Although urine can usually be readily collected from *Rh. harrisi,* the amounts collectable reflect the storage capacity of the bladders rather than rate of formation. Attempts to cannulate these small crabs have not been successful. Although several workers have estimated urine production in crustaceans by blocking of urinary pores and noting the consequent weight increase, the method has not proved satisfactory. Not only are the pores difficult to block (see below), but it is difficult to dry crabs consistently before weighing, resulting in imprecision. Further, there is the probability that obstructing urinary outflow prevents by back pressure the normal production of urine. Finally, in my experiments, even where urinary blockage was achieved and weight increases noted, no crab survived a weight increase of over 5%. Since such fatal increases took place in 10–20 hours in 10% sea water, one can conclude only that urine production in such a medium exceeds 5–10% of the body weight per day, and that the ability to release urine is of great physiological importance to the animal. Consequently a more complex and tedious but physiologically less drastic method was employed.

Crabs from a given medium (10% or 50% sea water) were individually numbered and weighed (after drying by wrapping in a towel and shaking). For each crab, a beaker of glass-distilled water was prepared, in volume 50 ml. per gram wet weight of crab. A seal of beeswax was applied to each crab with a warm cautery needle, completely covering the orbits and the sockets of antennae and antennules, but leaving the urinary opercula exposed. For each crab a series of three 50-ml. portions of distilled water was set up in small beakers. At timed intervals, each animal was passed through these 3 washes of distilled water to remove external salts, the total time in these preliminary baths being 5 minutes, at the end of which the crab was transferred to the test bath of glass-distilled water, the volume of which was known and in proportion of the animal's weight. After one hour in this first test exposure (A) each crab was returned to the adaptational medium, and a sample of the test medium saved for chloride analysis. A second set of test media (B) of glass-distilled water, of the same volumes as set (A), was then prepared, as well as fresh sets of the three 50-ml. washes. Each crab was then dried off, and the beeswax seal extended to cover its urinary pores. The elaborate sealing procedure was necessitated by the fact that two deep sutures at the sides of the movable antennal base interrupt the rim of each urinary pore and communicate with the areas about the bases of eyestalks, antennae, and antennules; weight increases were not observed in the earlier attempts to estimate urine production by blockage unless the seals were thus extended. Each "fully sealed" crab was then washed through 3 changes of distilled water and exposed for a second hour

TABLE 1

Urine volumes and urinary salt losses

Adaptational media	10% SW	50% SW	Pooled
Number of animals	16	15	31
Mean wet weight (grams)	1.82	1.66	1.74
Mean Cl-loss per gram of crab (m*M*)			
"A"	.0067	.0099	—
"B"	.0049	.0075	—
"C"	.0078	.0113	—
% of Cl lost via urine			
Range	−4.84 to 87.1	−7.14 to 78.2	−7.14 to 87.1
Mean ± S.D.	35.7 ± 24.6	34.0 ± 23.2	34.9 ± 23.5
Urine, % body weight ± S.D.			
Range (per hour)	−0.62 to 2.17	−0.30 to 2.07	−0.62 to 2.17
Mean (per hour)	0.97 ± 0.69	1.05 ± 0.75	1.01 ± 0.71
Mean (per day)	23.3 ± 16.6	25.2 ± 18.0	24.2 ± 17.0

(B) to glass-distilled water, at the end of which period it was again returned to its adaptational medium and water samples (B) taken. Next, the wax seal was removed from the urinary opercula of each crab, and a third series (C) of the 50-ml. washing baths and measured proportionate volumes of glass-distilled water prepared. Each crab was washed as before and exposed to distilled water for a third hour (C), at the conclusion of which each crab was removed and samples of the test media taken. Chloride concentrations of all test media (A, B, and C) were determined, and the m*M* of chloride lost per gram of crab determined. The chloride loss in the second hour (B; urinary pores blocked) was subtracted from the average loss in the first and third hours (A and C; urinary pores open). The difference was taken to represent that part of the total chloride loss which normally occurred *via* the urine. (The probable loss of some chloride through the thin membranes of the occluded orbital and antennal areas has been ignored.) The values obtained (see Table I below) indicate that about one-third of the total chloride loss in the experimental situation is *via* the urine, the fraction not being greater in the crabs adapted to 50% sea water than in those adapted to 10% sea water before the test.

If we may assume that in the above experiment the crabs continued to produce blood-isotonic urine, and that the one-hour exposures, separated as they were by restorative periods in the adaptational media, did not greatly alter the blood concentration, then it should be possible to calculate the volume of urine produced in one hour by using the relationship:

$$\frac{\text{Volume of urine}}{\text{Volume of test medium}} = \frac{\text{Concentration of urinary Cl}^{-}\text{ in test medium}}{\text{Cl}^{-}\text{-Concentration of urine}}$$

The above assumptions were tested by exposing a number of crabs (previously adapted to 50% sea water) in a large excess volume of glass-distilled water, changed at intervals, and testing the blood and urinary chloride concentrations of

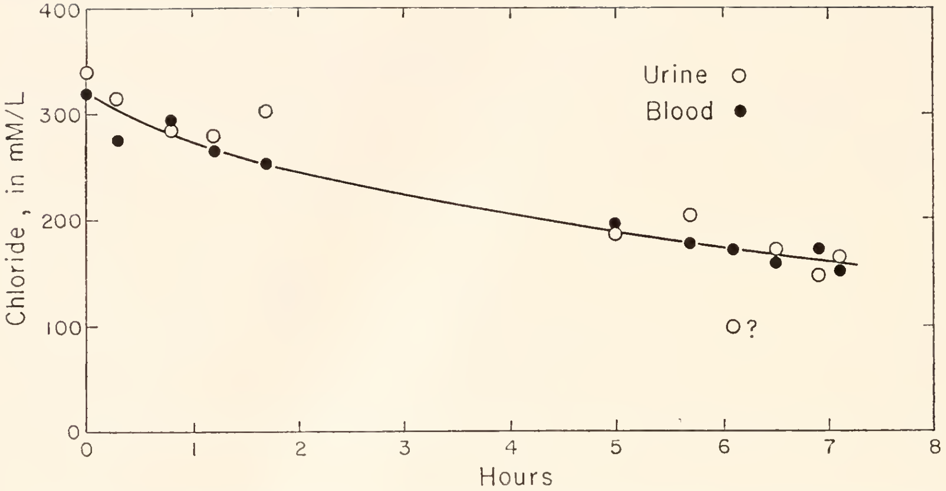

FIGURE 3. Chloride concentration of blood and urine during a 7-hour exposure to distilled water (after 50% SW), each pair of points based on a single individual. Dots, urine; circles, blood. Each point the mean of two samples.

individuals at times up to 7 hours. Figure 3 shows that the urine remains essentially isotonic (blood as usual shows the lesser chloride concentration attributed to its protein content), and that in one hour the drop in chloride concentration is only about 10%. It has therefore been assumed, on the basis of Figures 1 and 3, that blood (and consequently urine) chloride concentrations were 300 mM/L. in crabs adapted to 50% sea water and 250 mM/L. in those from 10% sea water. It is also assumed that the chloride losses in the test exposures were largely made good during the more-than-one-hour periods in the adaptational media which separated test periods A and B from B and C. The results of calculations based on the above assumptions indicate (Table I) that *Rh. harrisi* in the emergency of exposure to fresh water is capable of producing a quantity of urine approximately 1% of its body weight per hour, or 24% per day. Again there is no indication that crabs adapted to 50% sea water eliminate more urine in the test than do crabs from 10% sea water, despite the difference in the osmotic stresses or gradients. Twenty-four per cent of the body weight per day is undoubtedly in excess of that produced in most salinities, and probably tends to exceed the output in the lowest salinities met in nature. Figure 3 indicates that a 50% lowering of the chloride concentration of the blood can occur in 7 hours. This salt loss represents an adaptive lowering of internal osmotic pressure, significantly reducing the stress imposed by externally reduced salinity. It is evident that urine production, while accounting for one-third of the total salt loss, is at the same time of adaptive value in lessening osmotic swelling.

The above conclusions are only general approximations, since the method employed does not exclude several sources of error; *e.g.*, (1) Crabs might discharge urine during handling (observed) or washing prior to exposures A and C, and then might not release urine during these periods. (2) Crabs might release urine during A and/or C which had been stored up prior to the exposure and which rep-

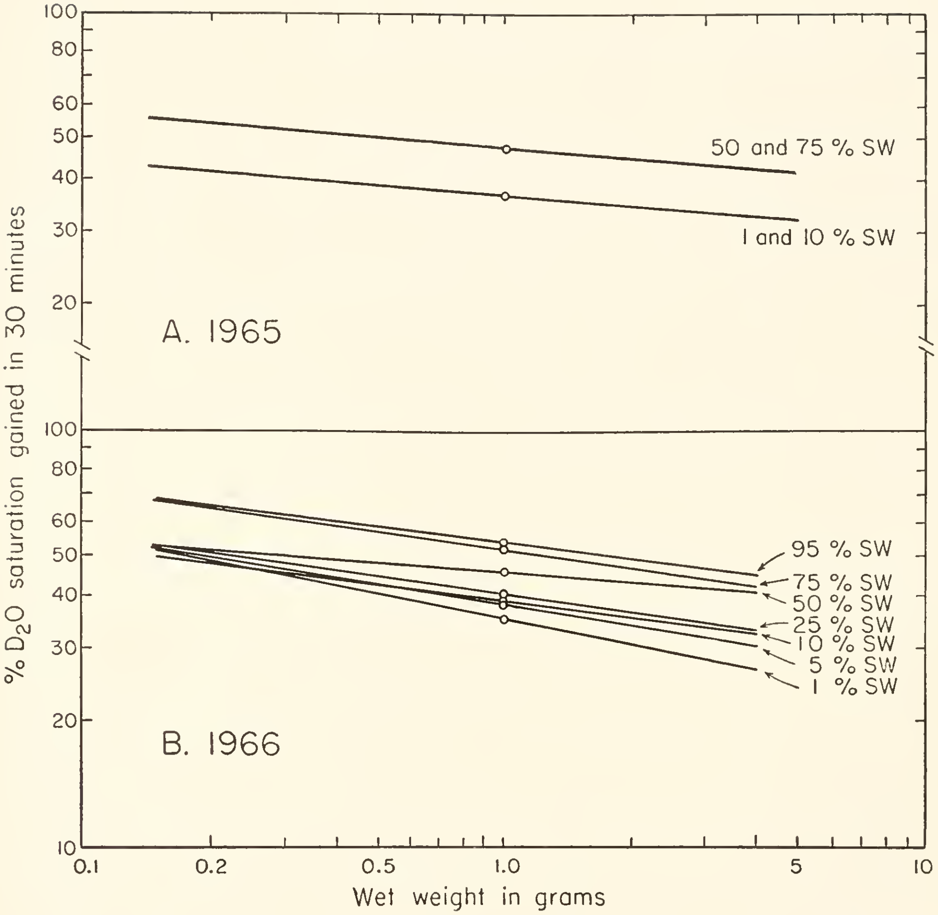

FIGURE 4. D_2O uptake as a function of body weight and salinity (as % sea water); A, 1965; B, 1966. Curves calculated by method of least squares on assumption that: Uptake = a•Weight to power (b − 1). Data in Table II.

resented more than an hour's accumulation. (3) Crabs might leak urine from beneath the seal, or might defecate during period B (despite the fact that crabs were not fed for several days prior to these experiments, defecation was observed in a few cases). The great scatter of the data, including even some "negative" values for urine production, suggests that such factors as enumerated above, in addition to experimental error, should dictate caution in accepting the results, but the mean values for urine production are reasonable and quite close to those for the urine of *Carcinus* in 40% sea water (Shaw, 1961a; pp. 144–145), and less than those of fresh-water crustaceans (except *Potamon*) cited by Potts and Parry (1964; p. 175). In one respect the data fail to reveal an expected relationship, namely, the volume of urine produced is not greater in animals adapted to 50% sea water prior to test than in those adapted to 10% sea water, although the greater osmotic gradient would seem to favor a greater intake and hence greater output of water in the animals from 50% sea water.

D. *Permeability to D_2O-entry as a function of external salinity*

The data presented above indicate that *Rhithropanopeus harrisi* can produce a copious urine under osmotic stress, hence under such conditions water must enter fairly readily. The methods used, however, do not indicate how much water enters under more normal conditions and, to determine the permeability of the animal to inward passage of water, tests have been made of the rate of entry of "heavy water" (deuterium oxide, D_2O) at various salinities with which the crabs were in steady-

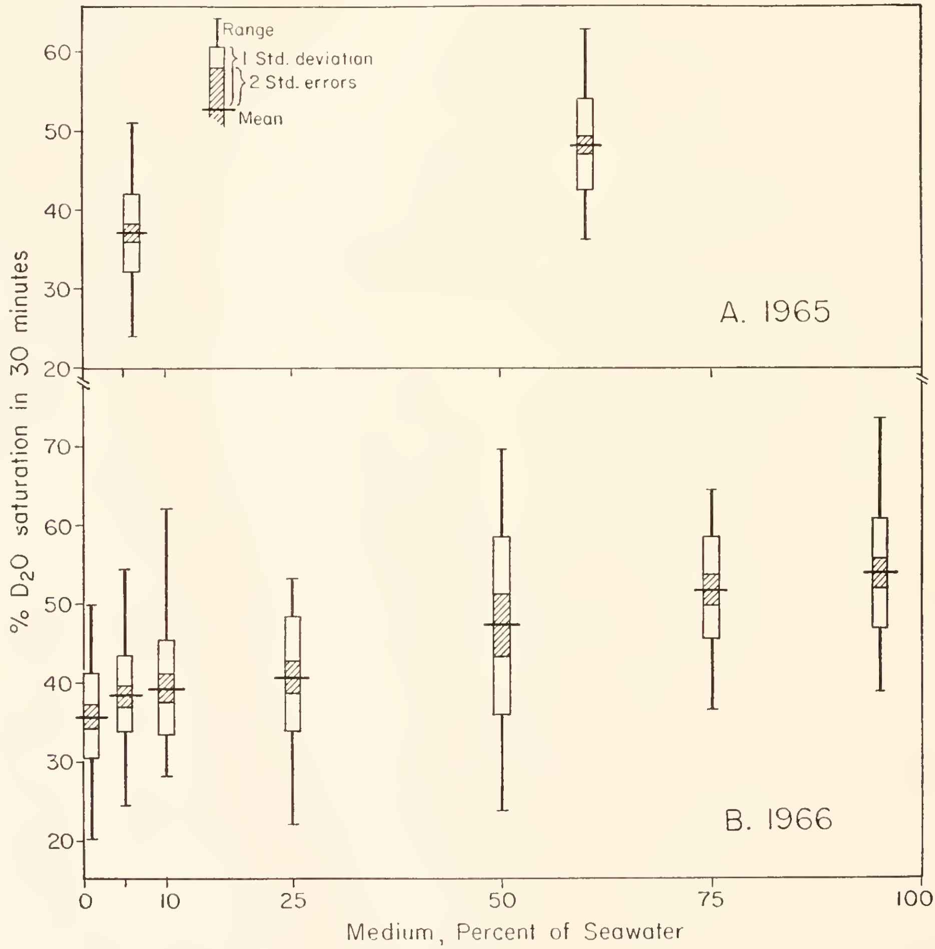

FIGURE 5. D_2O uptake as a function of salinity, corrected to body weight of 1 gram; A, 1965; B, 1966. Data in Table II. Plotted by method of Dice and Lerass (1936) (Contr. Lab. Vert. Genet., Univ. Mich., no. 3: 1–3), in which non-overlap of blocks representing ± 2 std. errors indicates significant difference.

Table II

D_2O-uptake (as % saturation gained in 30 min.) in relation to salinity (as % SW) and to body weight

% Sea water	Fig. 4: % sat = a Weight $^{(b-1)}$			Fig. 5: % sat. corrected to 1 gram weight			
	n	a	(b − 1)	Mean	Std. Dev.	Std. Error	*P*
1965 series							
1 + 10% SW	77	36.55	−.0802	37.0	± 4.94	±0.56	<.001
50 + 70% SW	92	47.68	−.0725	48.0	± 5.64	±0.59	
1966 series							
1% SW	46	35.32	−.1950	35.8	± 5.39	±0.80	<.025
5% SW	49	38.53	−.1596	38.4	± 4.79	±0.68	>.40
10% SW	47	38.90	−.1292	39.3	± 5.91	±0.86	>.20
25% SW	49	40.25	−.1452	40.9	± 7.20	±1.03	<.005
50% SW	30	45.79	−.0653	47.2	±11.14	±2.04	<.05
75% SW	43	51.88	−.1526	51.8	± 6.44	±0.99	>.10
95% SW	50	53.46	−.1363	53.9	± 7.06	±1.00	

state equilibrium. Preliminary tests showed that D_2O entry proceeded at a uniform rate for at least 3 hours (although a decrease later occurred, suggesting the presence of a second compartment) and that half-saturation of the blood required less than one hour. The method adopted was, therefore, to immerse weighed crabs for one-half hour in 40 times their weight of a 5 moles per cent D_2O solution made up at the salinity to which the crabs had been adapted (thus imposing no osmotic shock). At the end of the half-hour exposure, a blood sample was taken. Since D_2O uptake rate may be closely related to surface in one species, and more nearly proportional to weight in a related species (Smith, 1964), as wide as possible a range of sizes of crabs was employed (0.1 to 4 grams) and the D_2O uptake rate plotted against weight (Fig. 4). Uptake rate is expressed as the per cent of D_2O-saturation obtained in the half-hour exposure to D_2O made up in various percentages of sea water. In the first series of experiments, done in summer and fall of 1965, uptakes of 77 crabs in 1 and 10% sea water were not distinguishable; results were pooled, and the same was done with results obtained on 92 crabs in 50 and 75% sea water. Two conclusions are suggested by the curves in Figure 4A: First, the low slopes (−.0725 and −.0802) indicate that body size (weight) has but a small effect upon uptake rate; absolute uptake is more closely related to body weight than to surface. This means either that the permeability of larger crabs is relatively increased or, more likely, that the permeable areas (such as gills?) show a relative increase with increased body size, such that for purposes of water-entry the surface/volume ratio is kept nearly constant. Secondly, the levels of the curves in Figures 4A and 5A show that the entry of water (as represented by D_2O) is significantly lower (t test) at lower salinities (1–10% sea water, in the regulatory range) than it is at higher salinities (50–75% sea water, in which little if any regulation takes place). This second result was unexpected, since it might be presumed that the osmotic gradient of crabs regulating in low salinities would cause a greater water influx than in crabs in equilibrium with media of higher salinity.

In order to confirm the decrease in water (D_2O) permeability indicated in the 1965 experiments, a more extensive experiment was carefully carried out in the summer of 1966, using a total of 315 crabs from the same locality, adapted at 13° C. to a series of dilutions of sea water (1, 5, 10, 25, 50, 75, 95%), following closely the methods used in 1965. The results, summarized in Table II and plotted in Figure 4B, indicate that the decrease in D_2O permeability with decreased salinity is gradual over the range from 95% to 1% of sea water. The slopes $(b - 1)$ of the weight-uptake curves, averaging -0.1404 (-0.0653 to -0.1950), are greater than those obtained in 1965, but are still much less than the value (-0.3333) indicative of the "surface rule," *i.e.,* they still suggest that there is a tendency to compensate for increase in body size by some relatively greater increase in permeable surface. In order to assess the significance of the values obtained, the D_2O uptake rate of each crab was corrected to that of a crab weighing one gram, utilizing the $(b - 1)$ values for each salinity group. Figure 5 and Table II show the ranges, standard deviations, and standard errors of the 1965 and 1966 data so corrected. The D_2O uptakes are significantly different at better than the 1% level (t test) between 1%, 25%, 50% and 95% sea water, but the change is gradual enough to be less than significant between most adjacent salinities in the series. The probability that the series as a whole is due to chance is so low that the results in 1966 may be considered highly significant in a statistical sense, but what the significance is in a physiological sense is a more difficult problem.

How a reduction in permeability occurs (or if it occurs) is not clear. Conceivably there could be a reduction in permeable *area* rather than reduction in water permeability *per se,* but it seems more reasonable to seek a physiological mechanism for altering permeability than some morphological change of permeable area. It should be noted that these crabs were adapted to the salinities used in the above experiments for periods of 1–3 weeks, so that the differences are not the result of a long-term ontogenetic conditioning, nor are they consequent upon a molt. However, the minimum time required to effect a measurable water-permeability lowering has not yet been determined.

E. *Water content*

The water content of *Rh. harrisi* was estimated for a group of 15 crabs (wet weights 2.05 to 4.04 g.) adapted to 10% sea water and for a second group of 14 crabs (wet weights 2.05 to 4.14 g.) adapted to 50% sea water. Each group included two females and all animals possessed complete sets of legs and chelipeds. The size range was typical of that of crabs used in the estimation of urinary chloride loss in section C above. Crabs were shaken free of water in a towel, weighed individually, and dried at 95–100° C. Crabs from 50% sea water had a mean water content of 65.6%; those from 10% sea water averaged 65.2%. This constancy is not surprising, since 10% and 50% sea water bound the flattest part of the curve of regulation (Fig. 1).

Discussion

This survey of osmotic performance shows that *Rhithropanopeus harrisi,* as is usual for crabs, produces blood-isotonic urine, the volume of which (24% of body weight per day in an acute exposure to fresh water) is less than that produced by

Carcinus in 40% sea water (Shaw, 1961a; pp. 144–145), more than the production of *Eriocheir* in fresh water (Potts and Parry, 1964; p. 175), and vastly greater than the urine production of *Potamon* in fresh water (Shaw, 1961b). *Rhithropanopeus,* with its rather copious urine production, does not seem to have evolved significantly toward the greatly lowered water turnover rate and low water-permeability reported for *Potamon.*

However, *Rhithropanopeus* exhibits another capability, that of lowering its water-permeability as an adaptive response to a lowered external salinity. This is the first report in crustaceans of a phenomenon that has previously been detected in the brackish-water polychaete annelid *Nereis diversicolor* by Jørgensen and Dales (1957), who showed that worms in fresh water had a water-permeability not greater than 40% of that shown in 11% sea water. Smith (1964) likewise reported a possibly lowered D_2O influx when *Nereis succinea* and *Nereis limnicola* were tested in 5% sea water or fresh water, respectively, although the significance was doubted. The present demonstration of this phenomenon in *Rh. harrisi* raises the probability that environmentally-induced changes in water-permeability of body surfaces may be a more general phenomenon than has hitherto been assumed. It is compatible with the possibility, although it is not a proof, that the apparent low water-permeability of *Potamon niloticus* is an evolutionary refinement of a mechanism present more generally in cancroid Brachyura. It also serves to emphasize that, in discussions of permeability, one must specify to what the permeability applies, since water-permeability may vary independently of permeability to ions (Smith, 1964) or other substances (Leaf, 1965).

On the basis of the D_2O entry rates obtained under natural conditions of stress (10% and 70% sea water are well within the normal salinity variation met by *Rhithropanopeus* in nature) a calculation of the amount of water entering, and presumably available for elimination as urine, may be made by making certain assumptions; the data available seem inadequate for a close correction for back-diffusion. In *ca.* 70% sea water the blood is isotonic to the medium (Fig. 1) and both would have a salt concentration equivalent to 350 m*M*/L. NaCl (0.70 osmoles). Water concentrations inside and out are equal, namely 55.5–0.7 = 54.8 osmoles, and there should be no *net* osmotic inflow. In this situation we observe (Fig. 4A) that D_2O diffuses in at such a rate that 47.7% of the external concentration is reached in 0.5 hour. The water content of the crab is 65%, so that for a 1-gram crab, if we assume all H_2O in the medium is replaced by D_2O (50.0–0.7 = 49.3 osmoles) the diffusional influx of water (as D_20) would be expressed by 0.477 × 650 mg./0.5 hours = 620 mg./hr. This influx is balanced by an equal outflux. The influx per mole of external D_2O concentration would be 620 mg. per hr./49.3 moles = 12.6 mg. of water per osmole concentration difference per hour.

If the above 1-gram crab were to be placed suddenly into distilled water (concentration = 55.5 osmoles) there would be a concentration difference of 55.5–54.8 = 0.7 osmoles, producing a *net* water influx of 0.7 × 12.6 = 8.8 mg. per hour per gram of crab. This calculated value of 0.88% of the body weight is not far from the value of 1.05% of body weight per hour estimated in section C as the urine production of crabs adapted to 50% sea water and tested in distilled water. Indeed, a rough calculation of back-diffusion would indicate that diffusional input equals urinary output.

In *ca.* 10% sea water the blood has a salt concentration approximating 240 mM/L. NaCl (.48 osmole), and a water concentration of 55.50–.48 = 55.02 osmoles. The medium has a salt concentration of only *ca.* 50 mM/L. NaCl (.10 osmole) and a water concentration of *ca.* 55.4 osmoles. D_2O is observed (Fig. 4A) to diffuse in at such a rate that 36.6% of the external concentration is reached in 0.5 hour. The water content may also be assumed to be 65%. Thus for a 1-gram crab, if we assume all H_2O in the medium to be replaced by D_2O (50.0–0.1 = 49.9 osmoles) the diffusional influx of water (as D_2O) would be 0.366 × 650/0.5 = 475.8 mg./hr. The influx per mole of external D_2O concentration would be 475.8 mg. per hr./49.9 osmoles = 9.52 mg. of water (as D_2O) per osmole concentration difference per hour. But in this second instance the crab is not presumed to be in osmotic equilibrium with its medium, there being a water concentration difference of 55.40–55.02 = 0.38 osmole. This should produce a net influx of 0.38 × 9.52 = 3.62 mg. of excess water entering per hour in 10% sea water to be disposed of as urine. When such a crab is placed in distilled water the water concentration difference between medium and blood rises to 55.50–55.02 = 0.48 osmole, which should produce a net water influx into a 1-gram crab of 4.60 mg./hr. This calculated value of 0.46% of the body weight is only about half the value of 0.97% of body weight per hour in urine production estimated in section C for crabs adapted to 10% sea water before being tested in distilled water. An estimate allowing roughly for back-diffusion would give a value of 0.54% of body weight, still quite low.

In summary, the diffusional net influx of water (as estimated from D_2O influx) may not be enough to account for the urine produced under conditions when an osmotic influx might be expected to increase the urine flow.

That urinary output of water occurs even in the absence of an "osmotic" inflow resulting from a water-concentration difference has been so well known that it is usually not commented upon. Shaw (1961a, p. 144) has remarked, "It seems quite clear that the water required for urine production in *Carcinus* in full-strength sea water is not taken up osmotically since often no osmotic gradient exists and one must suppose, therefore, that the water is absorbed by some active process . . ." This is quite in harmony with the present observation that urinary output in *Rh. harrisi* may be double the calculated diffusional net water input. The phenomenon is, indeed, rather general, having been first noted in the frog by Hevesy, Hofer and Krogh (1935). Koefoed-Johnson and Ussing (1953) and Ussing (1954) cite other examples and confirm the original findings. Ussing (1954) suggests a simple model combining inner diffusional areas in series with outer narrow channels or "pores" such that, given a small diffusional net influx, a flow is set up in the pores of sufficient velocity to block diffusion in the opposite or outward direction. Such a membrane system would act in respect to diffusion like a one-way valve. It could admit water or D_2O, but since the expected compensating outward diffusion is blocked by the phenomenon of flow in the pores, there would be a greater-than-expected retention of water inside, to be disposed of in the urine. Ussing's concept applied to a crab hyper-regulating in dilute sea water would imply that, instead of an active transport of water as suggested by Shaw, we may have a surface membrane system in which, given a small net inward diffusion of water, the resulting bulk flow inwards through pores or areas behaving like pores results in a net inward movement of water resembling an active transport. Probably it is not that simple, but the epithelium-cuticle system of crustaceans deserves to be examined critically for direct evidence of differential water-permeability, as Ussing's hypothesis might suggest, or for active water transport as suggested by Shaw.

A further point for discussion is raised by Lockwood (1965, p. 68), who has made the generalization, based on studies of hypotonic urine formation in brackish-water amphipods, that, ". . . the conservation of ions within the body by the production of hypotonic urine is likely to be found to be a common feature of the smaller brackish water crustacea, especially those with a high rate of water turnover." On the basis of this interesting possibility, I have attempted to sample urine from smaller individuals of *Rhithropanopeus,* and am able to state that animals of less than one gram weight show no sign of hypotonicity in their urine, despite their high water turnover. It seems most unlikely that still smaller juvenile members of the population, comparable in size to Lockwood's amphipods, produce hypotonic urine and then give up this physiologically advantageous habit. It is to be noted that Lockwood has shown 80% of the salt loss in *Gammarus duebeni* to be *via* the urine. Probably Lockwood's generalization does not apply to crustaceans which, like *Rhithropanopeus,* suffer the major part of their salt loss *via* the body surface, and his prediction might be better stated as: ". . . the conservation of ions within the body by the production of hypotonic urine is likely to be found in those brackish and fresh water crustaceans which combine a high rate of water turnover with a significant reduction in the salt-permeability of the body surface." Whatever the outcome, Lockwood's stimulating generalization should be tested upon a wide variety of crustaceans.

I am indebted to Miss Etta Kwan for permission to use in Figures 1 and 2 certain data from her senior honors thesis research. The data in Figures 4B and 5B and in Table II (1966) were largely the result of the conscientious technical assistance of Miss Georgiandra Little, for whose care and skill I am most grateful.

SUMMARY

1. The osmotic performance of the small cancroid crab *Rhithropanopeus harrisi* (Brachyura) has been surveyed in order to assess the mechanisms mainly responsible for its success in colonizing waters of low salinity.

2. This crab shows hyper-regulation of chloride and osmotic pressure in media up to about 60–70% sea water, and a slight tendency to hypo-regulate in higher salinities.

3. Like other crabs, *Rh. harrisi* maintains a relatively high blood concentration and produces a blood-isotonic urine.

4. Urine production, estimated by an indirect method, approximates 24% of the body weight per day in low salinities, implying a high rate of water turnover.

5. Approximately $\frac{1}{3}$ of the total salt loss is *via* the urine.

6. Inward permeability to water, as judged by D_2O influx rate, is decreased at lower salinities. This mechanism, here demonstrated for the first time in crustaceans, is suggested as being of adaptive significance.

7. Urinary output of water exceeds the diffusional net (osmotic) input of water as calculated by D_2O influx, suggesting the possibility that a differential diffusional permeability to water or some form of non-diffusional water transport may be involved.

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Note added in proof: Since this paper went to press, a pertinent paper by Rudy (1967) has appeared, in which he states that the brackish-water crab *Carcinus maenas* and prawn *Palaemonetes varians* "cannot significantly alter their integumental water permeability." A re-expression of my data in terms approximating Rudy's "H_2O influx constant" (per cent of body water exchanged per hour) and the extrapolation of my weight/uptake curves to give values for 40-gram *Rhithropanopeus* (if such existed) lead me to conclude that no necessary incompatability between our claims exists. *Carcinus* possibly exhibits the same tendency to altered water-permeability as does *Rhithropanopeus,* although perhaps not (on the basis of limited data) to a "significant" extent.

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THE PHOTOSYNTHETIC RHYTHM OF ACETABULARIA CRENULATA. I. CONTINUOUS MEASUREMENTS OF OXYGEN EXCHANGE IN ALTERNATING LIGHT-DARK REGIMES AND IN CONSTANT LIGHT OF DIFFERENT INTENSITIES[1]

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Until recently, most investigators of biological rhythms have concerned themselves with drawing inferences about the properties of the intrinsic timing mechanism from measurements of some physiological or developmental process. The lack of any more direct experimental approach has largely frustrated attempts to achieve an understanding of the "clock" at the molecular level.

This paper presents continuous records of the rate of oxygen exchange by *Acetabularia* cells over periods of several days to one week. A recently developed polarographic system employing a graphite cathode was used to monitor oxygen metabolism. During the experiments pretrained cells were exposed to alternating light-dark regimes and to continuous light of different intensities. A second paper seeks to reveal the point at which control is exercised by analyzing the time-dependence between the rate of photoassimilation of CO_2 and the activity of selected enzymes in the reductive pentose phosphate pathway (Hellebust, Terborgh, and McLeod, 1967).

The unicellular alga *Acetabularia* possesses a number of properties which make it a particularly suitable subject for an investigation of the means of control of a rhythmic process. It exhibits a prominent circadian rhythm of photosynthesis expressed both in CO_2 uptake (Richter, 1963) and in O_2 evolution (Schweiger *et al.,* 1964) which persists under constant conditions, even in the absence of its nucleus (Sweeney and Haxo, 1961). Of further relevance are the facts that it grows by cell enlargement without any apparent differentiation for a period much longer than that required for expression of the rhythm, and that measurements on single cells are technically simple (Schweiger *et al.*, 1964).

MATERIALS AND METHODS

Experimental material consisted of groups of intact *Acetabularia crenulata* Lamarous cells in the phase of stalk elongation (Terborgh and Thimann, 1965).

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Cultures were maintained in a rapid state of growth in cotton-stoppered 500-ml. Erlenmeyer flasks containing Erdschreiber solution. Unless otherwise stated, cultures were given an alternating, 8-hour-16 hour, light-dark regime for at least two weeks prior to experimentation. Illumination of 300 ft.-c. was provided by "Daylight" fluorescent tubes during the light periods. The temperature was held at approximately 28° C. throughout the training cycles and during experimentation.

The rate of oxygen exchange by samples of 20 to 50 cells of 0.5 to 1.0 cm. in length was measured by means of the open polarographic system described by McLeod *et al.* (1965). The device measures the relative oxygen tension in a cup-shaped sample chamber 1 cm. in diameter. The chamber is lined below with a graphite paste that serves as the cathode in the oxygen reaction at a polarizing potential at 0.64 V.

The cathode chamber was covered above with a transparent dialysis membrane that held the algal sample in place, the whole device being immersed in a bath of Erdschreiber medium that doubled as an electrolyte. The anode was a Ag-AgCl bar located in the bath 3 cm. from the cathode chamber. Current flow between the electrodes was proportional to the oxygen tension in the cathode chamber. Signals fell in the range of 0 to 2 μamps and were amplified by a General Radio Model 1230-A electrometer and recorded continuously on a Varian G-14 or a Sargent SR recorder operated at very low chart speeds.

Readings obtained from a sample of cells in the dark reflect the concentration of oxygen in the cathode chamber during respiration. Since the solution around the sample is allowed to exchange freely with the bath, any constant rate of metabolic activity will in time establish a steady-state balance between the rate of oxygen exchange in the tissue and the rate of oxygen diffusion through the dialysis membrane. After major (on-off) changes in the illumination of the cells, complete establishment of a new steady-state of gas exchange requires $\frac{1}{2}$ to 1 hour, though about three-quarters of the change takes place in the first 5 to 10 minutes. Illumination of an algal sample thus results in a sharply rising trace.

The electrode assembly was housed in a light-tight wooden box which had a slide projector mounted outside on the end wall. The collimated beam was directed into the cathode chamber containing the algal sample by means of first-surface mirrors. Its intensity was controlled witht a variable transformer. Readings from a GE foot-candle meter (Model No. 213) at a level comparable to that of the sample chamber under appropriate layers of Erdschreiber medium and dialysis membrane were taken as estimates of the intensity incident on the algal samples at different Variac settings. Further details, including a diagram of the electrode assembly and optical system, are given by Terborgh (1966).

RESULTS

Oxygen metabolism of Acetabularia crenulata in alternating periods of light and darkness

Continuous records of oxygen exchange by groups of *Acetabularia* cells in an alternating light-dark (LD) regime reveal a complicated time course in light that consists of two peaks of photosynthetic output (Fig. 1).

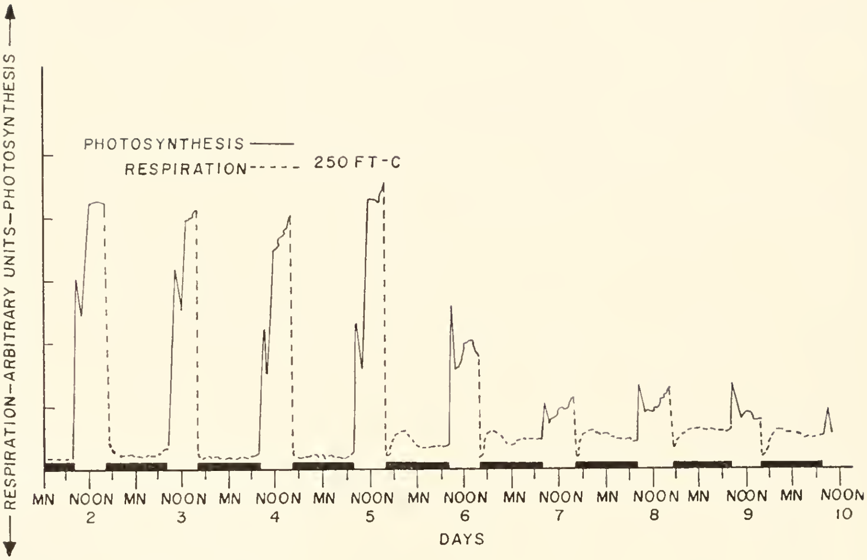

FIGURE 1. Oxygen exchange by *Acetabularia* cells in a 24-hour regime composed of 8 hours of light at 250 ft.-c. and 16 hours of darkness. Ambient temperature was 28° C. Heavy lines on abscissa denote dark periods. Decreased performance after day 5 was attributed to a gradually increasing toxicity of the electrode environment.

The first (stage 1) appears immediately on illumination and in approximately 30 minutes is followed by a sharp depression. A second rise (stage 2) begins after about 1 hour and develops a higher maximum after 4 to 8 hours.

This second upswing in the traces continues for the remainder of the light period. The prolonged gradual rise that is observed in this portion of the records is slow in relation to the response time of the measuring system, and so represents closely the actual time course of the photosynthetic output of the sample. Maximum rates were generally attained in the latter part of the daily light periods, often in the final hours.

On extinguishing the light, the concentration of oxygen in the sample chamber dropped rapidly for about 30 minutes and then decreased gradually until a respiratory steady-state had been established. Nearly steady traces were the rule during 16-hour dark periods in phase with the entraining regime.

The electrode environment did not appreciably affect the performance of algal samples for the first 4 or 5 days of recording. Subsequent progressive decreases in both photosynthetic and respiratory activity indicated the onset of deleterious conditions, possibly resulting from a gradual accrual of toxic levels of silver ion in the medium.

Partial specificity of this inhibition was apparent in that all parts of the cycle were not equally affected. The loss of activity in stage 2 subsequent to day 5 in Figure 1 was much more pronounced than the reduction of stage 1, the maximum photosynthetic rate then being found in the latter. This result thus suggests that the two stages are inhibited by different processes.

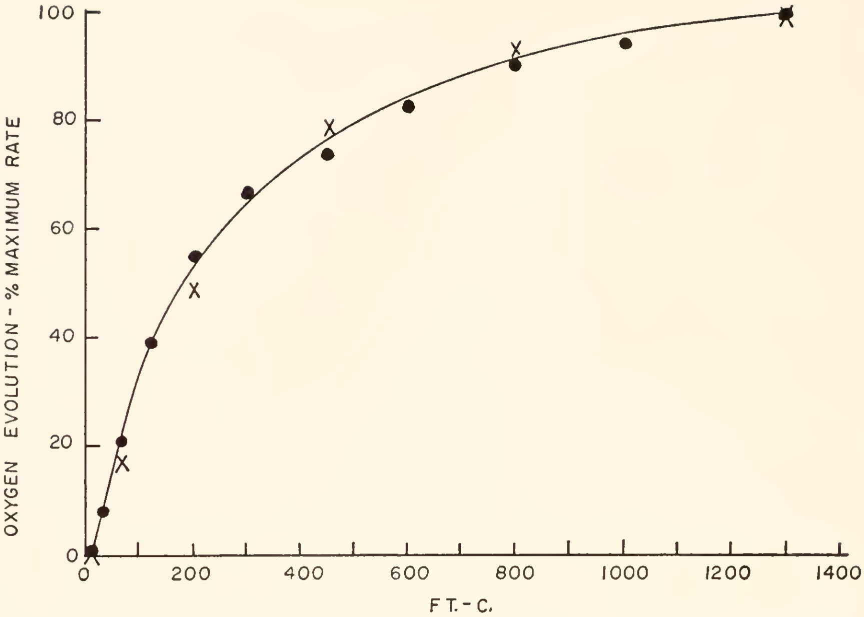

FIGURE 2. Photosynthesis as a function of the intensity of light of *Acetabularia* cells grown in a regime of 8 hours of light at 300 ft.-c. and 16 hours of darkness. Experimentation was carried out in the latter half of a light period. Performance at each intensity was taken as the increase in rate of oxygen production recorded over 30 seconds of illumination after the cells had established a steady-state of oxygen exchange in the dark. O = increasing intensity series, X = decreasing intensity series.

The effects of the electrode environment may result in a much-altered time course of respiration in the dark periods. Under these conditions, the respiratory cycle assumes a form similar to that of photosynthesis, consisting of a transitory early maximum followed by a slowly developing second stage of more rapid activity. As was the case for oxygen evolution, the initial burst of respiratory oxygen consumption appeared to be less sensitive to inhibition than the later stage. The first period of inhibited respiration came after the fifth day of the experiment, during which photosynthesis was normal, indicating that substrate limitation was probably not the cause.

The light curve of Acetabularia crenulata

Endogenous regulation of a photosynthetic rhythm could be exercised at the level of either the light or dark reactions, or alternatively through alterations in the tightness with which the light and dark reactions are coupled. One may distinguish between at least the first two of these possibilities by determining the effect of different light intensities on the expression of the free-running rhythm. The interpretation of such experiments is based on the characteristics of a photosynthetic light curve such as the one presented in Figure 2. The cells used in producing the curve were grown in an 8L–16D regime and subjected to experimentation in the latter half of a light period. The physiological state of the cells was therefore that of late stage 2 when the diurnal photosynthetic cycle is at its maximum. Before each exposure to light the algae were allowed to reach a steady-state of oxygen exchange in the dark. The rate of photosynthesis at each intensity was then taken as the increase in the rate of oxygen production that took place during 30 seconds of illumination. Since we observed stage 1 responses only following dark periods of at least several hours' duration, the light curve in Figure 2 is attributable to stage 2 photosynthesis.

The light curve obtained in this manner has an I_k somewhat below 300 ft.-c. whereas saturation required intensities in excess of 1000 ft.-c. I_k is the intensity at which the extrapolated initial slope of a light curve intersects a horizontal line through the maximum rate of photosynthesis. Curves of this form are produced even by single *Acetabularia* cells (unpublished results), eliminating the possibility that mutual shading was responsible for the unusually slow approach to saturation. Consequently, the rate of photosynthesis of *Acetabularia* over a wide range of intermediate light intensities is not limited solely by a light reaction or a dark reaction, *sensu strictu.*

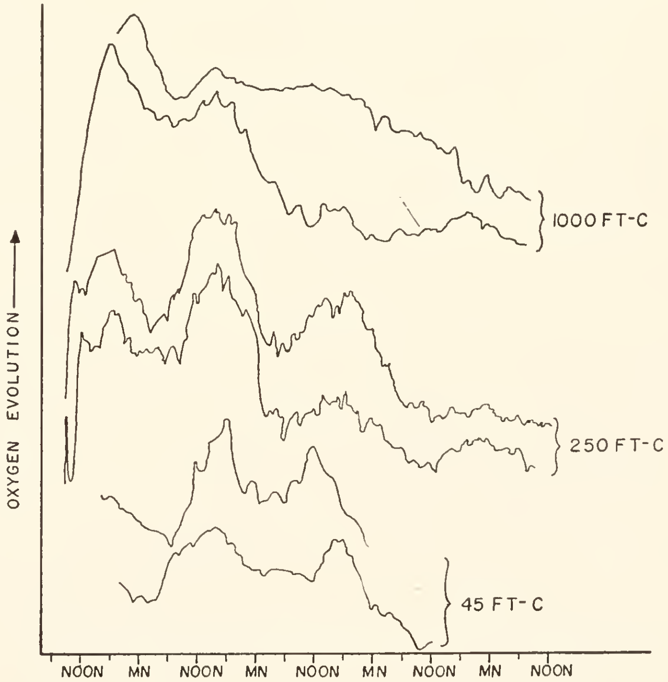

FIGURE 3. The free-running rhythm of oxygen production in continuous light at three intensities. Scale of ordinate is non-uniform. Prior to experiments the cells received 8 hours of illumination at 300 ft-c. daily from 9 to 17 o'clock.

Stages 1 and 2 of the diurnal cycle at different light intensities

Having shown that stages 1 and 2 of the diurnal photosynthetic cycle appear to be limited by different processes, an attempt was made to further characterize them by testing the response to step-up increases in light intensity at different times in the cycle. Estimates of the maximum photosynthetic capacity were obtained at intervals throughout the day by raising the light intensity from 250 to 1000 ft.-c. for periods of 90 seconds. The time course of photosynthesis revealed by the high-intensity spot checks essentially paralleled that observed at 250 ft.-c. However, the relative rate increases stimulated by the 1000 ft.-c. irradiations were appreciably greater during the maxima of stages 1 and 2 than during the depression between them. At this time the capacity of the cells appears to have been reduced to a greater extent than their activity.

Expression of the free-running rhythm in continuous light of different intensities

Regular oscillations in the photosynthetic output of *Acetabularia* cells exposed to continuous light persist for many days. This circadian rhythm is conspicuously expressed over a range of light intensity that encompasses the three major sections of the light curve (Fig. 3). Preceding the beginning of a continuous light regime the cells received 8 hours of illumination at 300 ft.-c., followed by a dark period. Experiments began at the start of the subsequent light period at near saturation (1000 ft.-c.), at an intermediate level (250 ft.-c.), and at an intensity on the linear portion of the light curve (45 ft.-c.). At all three levels, but especially at the highest, the daily photosynthesis decreased during the course of the experiments. The electrode microenvironment may have contributed to this behavior, but it could also represent a physiological adaptation to continuous light in cells preconditioned to 8-hour photoperiods.

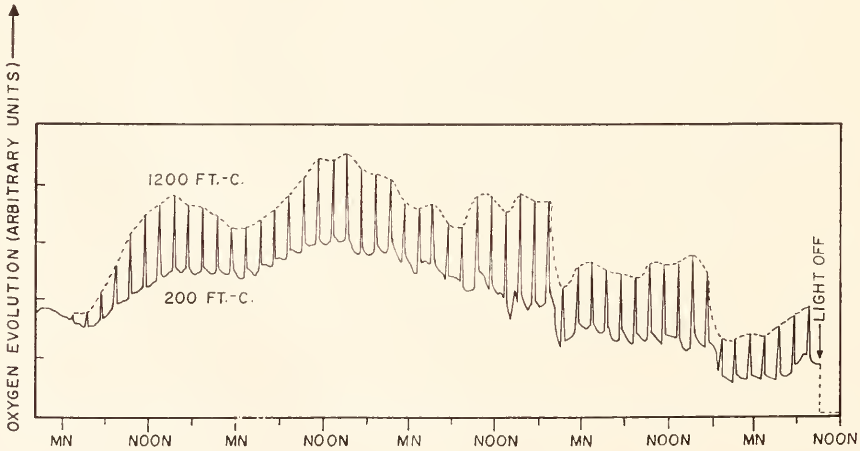

FIGURE 4. The free-running rhythm of oxygen production in continuous illumination of 250 ft.-c. on which 15-minute exposures to 1000 ft.-c. were superimposed every 2 hours.

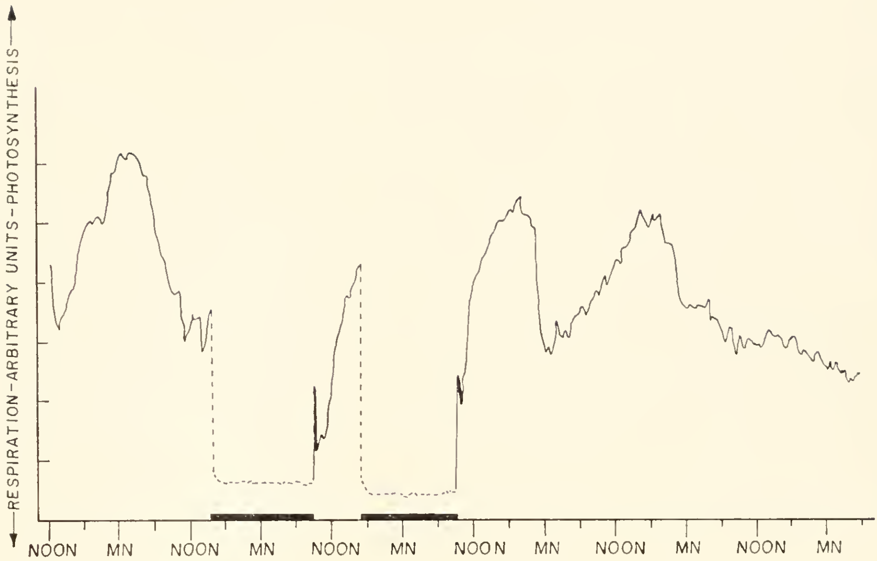

FIGURE 5. The effect of a single 8-hour light period on the phase of the rhythm of oxygen production. Prior to the start of the experiment the cells had been in continuous light for 1 week, during which the time of maximum output had shifted to 1 to 2 o'clock in accordance with a free-running period somewhat in excess of 25 hours.

The amplitude of the circadian oscillation diminishes rapidly under high-intensity illumination, but the vestige of the rhythm that remains after 3 or 4 days indicates that its period is unaffected. The free-running rhythm is exhibited with maximum amplitude at intermediate light intensities. In none of these experiments was there any indication that either the period or the phase of the free-running rhythm was affected by light intensity.

In order to establish that the photosynthetic apparatus was not adapting to whatever light intensity was being used to demonstrate the free-running rhythm, experiments were programmed to give indications both to the capacity and activity of photosynthesis throughout the cycle (Fig. 4). This was accomplished by giving 15-minute exposures to high intensity (1000 ft.-c.) illumination every 2 hours on a continuous background of moderate intensity (250 ft.-c.). The maximum rates recorded at the lower intensity did not exceed the minima in the high intensity cycle. This result clearly shows that the maximum photosynthetic capacity of *Acetabularia* remains well above the range of the oscillations in activity observed at low or intermediate intensities. It is evident that capacity and activity vary simultaneously, and that the former undergoes changes of greater amplitude.

Resetting the rhythm of photosynthesis

Figure 5 illustrate an experiment performed with cells that had previously been held in continuous illumination of 300 ft.-c. for one week after having been entrained with the usual 9 to 17 o'clock light periods. The first day's record shows that the free-running rhythm had persisted with undiminished amplitude through a week devoid of recurrent stimuli. The maximum in the cycle now came at 1 to 2 o'clock instead of at 17 to 19 o'clock, indicating the free-running period is not exactly 24 hours in constant conditions.

With the commencement of a second cycle of photosynthetic output, the cells were given 18 hours of darkness followed by a 9 to 17 o'clock training period. The free-running rhythm in 250 ft.-c. subsequently attained maxima around 18 o'clock in the manner of cells that had received many 9 to 17 o'clock entraining periods (*e.g.*, Fig. 3). A single light-dark cycle thus suffices to reset rhythmicity.

In the experiment of Figure 5 the response to illumination during the 8-hour training period was exceptionally poor even though it was given during a portion of the cycle when the photosynthetic performance should have been falling. Oxygen production in the entraining period did not reach the minimum level of the preceding or subsequent free-running cycles for 2½ hours. Depression of photosynthesis in out-of-phase light periods occurred to varying degrees in other experiments of this kind, though usually less than in the present instance.

Discussion

In constant illumination at 28° *Acetabularia* cells exhibit a free-running rhythm of oxygen production. Continuous records of the photosynthetic output over 8-hour light periods that were in phase with the entraining regime showed a time course consisting of two distinct stages. Upon illumination a rapid rate of oxygen production commenced immediately as indicated by the sharp initial rise of the traces. This is conspicuous only following periods of darkness of several hours, and thus may be dependent on the gradual accumulation of a rate-limiting substrate. The initial burst of oxygen production is followed by a depression and a subsequent slow rise to a second maximum (stage 2).

The gradual rise and fall of photosynthetic output in constant light follows kinetics similar to those of stage 2 of the diurnal time course and suggests that the two oscillations share the same mechanism. Since stage 1 is a direct consequence of long-continued darkness, it should not be confused with the manifestations of rhythmicity in stage 2 of LD schedules and continuous light.

That different reactions limit the rate of oxygen production during stages 1 and 2 is attested by their differential sensitivity to the deleterious effects of long-term exposure to the electrode environment. Investigation of the possible periodic expression of stage 1 would be of interest in reference to the two-component model for a temperature-compensated rhythm proposed by Hastings and Sweeney (1957).

The photosynthetic rhythm of *Acetabularia* differs from that of some other unicellular algae in being expressed, for several cycles at least, in continuous light at any intensity within the range of 45 to 1000 ft.-c. The rhythms of luminescence, photosynthesis and cell division in the marine dinoflagellate *Gonyaulax polyedra* are all promptly suppressed by light intensities of 800 to 1000 ft.-c. (Hastings *et al.*, 1961; Hastings and Sweeney, 1964). This result holds for photosynthesis assayed as carbon dioxide fixation (Hastings *et al.*, 1961) or oxygen evolution (Sweeney, 1960). In continuous dim light (100–200 ft.-c.) rhythmicity of luminescence and cell division persist in *Gonyaulax*. The rhythm, which involves the capacity for photosynthesis, is not observable until the capacity is tested by exposing the cells briefly to bright light (1000 to 1500 ft.-c.). Thus Hastings *et al.* (1961) concluded that only the maximum photosynthetic capacity of *Gonyaulax* was affected. Determination of light curves for oxygen evolution by single *Gonyaulax* cells at different times of day led to the same conclusion (Sweeney, 1961). Such a clear interpretation cannot be afforded the work of Palmer *et al.* (1964) who found that circadian oscillations in carbon dioxide fixation by the diatom *Phaeodactylum tricornutum* persisted for at least 3 cycles under continuous illumination at 80 ft.-c. but quickly damped at 20 and 600 ft.-c. Sweeney has shown that the activity of ribulose-diphosphate carboxylase in *Gonyaulax* extracts varies with the same period and amplitude as the rhythm of photosynthesis (1964, 1965).

When *Acetabularia* is brought into continuous light from an LD regime (8 hours at 300 ft-c. and 16 hours darkness in these experiments) the daily photosynthesis is at first high but tends to drop within two or three days to considerably lower levels. This is generally accompanied by a reduction in the amplitude of the rhythm and is particularly rapid and pronounced at high light intensity. To some extent this effect may be attributable to deleterious effects of the electrode environment, but in control experiments there was no reduction of photosynthetic output in LD until after the fifth day of experimentation. Thus the observed decreases in photosynthesis under continuous illumination may be largely adaptive.

This notion is supported by the results of earlier experiments in which cells given 8-hour photoperiods grew more slowly at both high and low intensities and contained up to 3 times as much chlorophyll as those given 16-hour photoperiods or continuous light (Terborgh and Thimann, 1964). It is apparent from these results that when *Acetabularia* is transferred from an 8-hour light regime to continuous light there follows a considerable reduction in chlorophyll content over the next few days. Thus the gradual loss of productivity that characterizes our records of the free-running rhythm, to a degree at least, must be attribuable to concomitant adaptive reductions in chlorophyll content. On the other hand, the possibility that diurnal fluctuations in chlorophyll content contribute to the expression of the photosynthetic rhythm has been thoroughly examined with the result that no such variations are discernible (Hellebust *et al.,* 1967). Adaptive changes in chlorophyll concentration in response to altered conditions require periods in excess of 24 hours in *Acetabularia* and so cannot account for any part of the photosynthetic rhythm. Reported diurnal cycles in the chlorophyll content of the leaves of a number of higher plants entail concentration changes of only 20% or less and therefore could not in themselves produce a photosynthetic rhythm of large amplitude (Wendel, 1957; Bünning, 1959). Parallel oscillations in photosynthesis and chlorophyll content of some natural phytoplankton communities (Yentsch and Ryther, 1957) are subject to a variety of interpretations (Steemann Nielsen and Jorgensen, 1962). Even in *Chlorella,* the adaptive adjustments in chlorophyll content that follow step-up or step-down changes in light intensity are much less rapid than those that would be necessary to mediate a circadian rhythm in photosynthesis (Steemann Nielsen *et al.*, 1962).

The fact that the photosynthetic rhythm of *Acetabularia* is expressed at both high and low light intensities does not permit a simple interpretation. One possibility is a time-keeping mechanism that separately and simultaneously controls rate-limiting steps in both the light and dark processes. Detracting from the credibility of this view, however, are the facts that *Acetabularia* undergoes no cyclic variation in chlorophyll content and that extensive experimentation has failed to reveal a potentially rate-limiting step in the Calvin cycle, though the activities of some 9 enzymes have been examined (Hellebust *et al.*, 1967). A simpler alternative would be a control system that operates at the biochemical level at which the light and dark processes are coupled. The notion of metabolic plasticity at this point can also be implied from the slowness with which the light curve reaches saturation. Linearity holds only to 150 or 200 ft.-c. in contrast with *Gonyaulax* in which it extends to 500 to 800 ft.-c. depending on the time of day (Sweeney, 1960). Clearly, more substantive evidence from this difficult area in metabolism is required before the rate-limiting reaction(s) can be pinpointed.

Our finding that a single 8-hour light period suffices to reset the phase of the photosynthetic rhythm in *Acetabularia* is in good accord with results obtained with other photosynthetic organisms. For instance, a 12-hour period of illumination resets the time of maximum phototactic responsiveness of *Euglena* (Bruce and Pittendrigh, 1956). Even more sensitive is *Gonyaulax* which can be rephased by a single exposure to altered light intensity. The number of hours of phase shift produced depends on both the intensity and duration of such treatments (Hastings and Sweeney, 1958).

We wish to thank Dr. Beatrice M. Sweeney for her thoughtful reading of the manuscript and for several helpful suggestions.

SUMMARY

1. Oxygen exchange by *Acetabularia crenulata* in various light regimes was monitored continuously for as long as 10 days by means of a rate-measuring graphite oxygen electrode.

2. The time course for photosynthesis in 8-hour light periods is bimodal, and consists of an initial burst of oxygen production followed by a depression and a subsequent slow rise to a (usually) higher maximum in the latter part of the period. The two maxima show differential sensitivity to the deleterious effects of long-term exposure to the environment of the oxygen electrode.

3. The light curve for photosynthesis departs from linearity at the comparatively low intensity of 200 ft.-c. but does not reach saturation below 1300 ft.-c., indicating an unusually loose coupling of the light and dark reactions.

4. Both maxima of the diurnal time course of oxygen evolution as well as the free-running rhythm were expressed at a moderate (250 ft.-c.) and at a high (1000 ft.-c.) light intensity. A free-running rhythm was also found at 45 ft.-c. The possibility that only the maximum capacity of photosynthesis fluctuates in the expression of the rhythm was ruled out in an experiment that monitored both capacity and activity alternately in the same cycles.

5. The natural period of the rhythm at 28° C. is approximately 25 hours. The phase can be reset by a single 8-hour photoperiod.

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THE PHOTOSYNTHETIC RHYTHM OF ACETABULARIA CRENULATA. II. MEASUREMENTS OF PHOTOASSIMILATION OF CARBON DIOXIDE AND THE ACTIVITIES OF ENZYMES OF THE REDUCTIVE PENTOSE CYCLE

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Research in biological rhythms has brought to light a large number of cases in which highly integrated physiological processes in both higher and lower plants are regulated periodically by an endogenous time-keeping mechanism. Recently a number of investigations have sought to reveal the point at which control is exercised by analyzing the time-dependence of key component steps. For example, Richter and Pirson (1957) found diurnal rhythms in the activities of acid phosphatase, catalase and phosphorylase in cell extracts of the green alga *Hydrodictyon* that correlated positively with diurnal changes in photosynthetic and respiratory rates. However, no such changes were discerned in the activities of aldolase and triosephosphate dehydrogenase, enzymes that are known to be more specifically involved in photosynthetic and respiratory pathways. Khan and Sanwal (1965) in studying diurnal fluctuations in the organic acid content of the cactus, *Nopalea dejecta,* discovered parallel periodicity in three enzymes of the tricarboxylic cycle.

Studies on the marine dinoflagellate, *Gonyaulax polyedra*, have been particularly interesting because of the apparent directness and simplicity of the mode of periodic control in this organism. Hastings *et al.* (1956 and 1962) showed that the rhythm in bioluminescence may be attributed in part to concurrent changes in the amount of luciferin and in the activity of luciferase in cell-free extracts. *Gonyaulax* also exhibits a marked circadian rhythm in photosynthetic capacity when cells are maintained in dim light and exposed periodically to a saturating intensity for brief intervals (Sweeney, 1960). The fact that no rhythmicity is expressed at rate-limiting light intensities suggested that dark reactions, only, are affected by the control mechanism. Subsequent investigation revealed that the Hill reaction of whole cells in the presence of quinone and the sensitivity to uncouplers of photophosphorylation do not undergo diurnal variation (Sweeney, 1965). On the other hand, the activity of ribulose diphosphate carboxylase in cell extracts varied in a diurnal cycle, the amplitude of which was sufficient to account for the observed rhythm in photosynthetic capacity. Sweeney's paper appears to be the first report of a close parallel in a photosynthetic rhythm and the activity of an enzyme known to be essential to the photoassimilation of CO_2, though the data unfortunately do not permit a quantitative comparison between the rate of photoassimilation and the activity of the enzyme.

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In the present work we have undertaken such comparisons using the slowly developing giant alga, *Acetabularia crenulata,* which is known to possess a pronounced photosynthetic rhythm (Sweeney and Haxo, 1961; Richter, 1963). Cells taken from the light and dark phases of a daily cycle have been compared in their ability to photoassimilate CO_2 at low and high light intensities and in the activity of 9 enzymes in the reductive pentose phosphate pathway. *Acetabularia* cells expand logarithmically for a number of weeks without cell division before undergoing morphogenesis (Terborgh and Thimann, 1964) and so possess the inherent advantage that their photosynthetic rhythm is uncomplicated by concurrent biochemical changes associated with differentiation and a cell division cycle.

Materials and Methods

The experimental material consisted of *Acetabularia crenulata* Lamoroux cells in the phase of stalk elongation. The conditions of growth were the same as described in the preceding paper (Terborgh and McLeod, 1967). The cultures were given a regimen comprised of alternating 8-hour light and 16-hour dark periods for at least two weeks prior to experimentation. The light source was a battery of "Daylight" fluorescent tubes that gave an illuminance of 3 klux at the level of the culture flasks. Experimental cells were conditioned in two chambers in which the light-dark cycles were 180° out-of-phase. Samples were collected simultaneously from the two chambers, and will be referred to as L (middle of light phase) and D (middle of dark phase) samples. Portions of the samples were then immediately used for measurements of photoassimilatory rates, fresh weight and chlorophyll determinations, and for the preparation of homogenates for enzyme analyses.

Measurements of photoassimilation employed approximately 50-mg. fresh weight portions of *Acetabularia* cells. These were suspended in square 4-ml. cuvettes containing 3 ml. of medium that held a known amount of $NaHCO_3$. The cuvettes were covered with Parafilm and exposed to illumination in a water bath at 25° C. The light source was a tungsten bulb, from which most of the heat was eliminated by passing the beam through a tank of water. The light intensity at the position of the cuvettes was varied by interposing neutral density filters, and/or varying the distance of the light source. At the beginning of the experiments 2 μC. of $NaHC^{14}O_3$ were added to the cuvettes, and after 15 minutes of photoassimilation of labelled bicarbonate the cells were removed from the cuvettes, rinsed in fresh medium, blotted lightly, and transferred to vials containing 5 ml. of methanol. The cells were then ground in a glass homogenizer with a small amount of glass-fiber paper added to facilitate the grinding. After homogenization, 25 μl. of 1 N HCl were added, and air was bubbled through the suspension for about three minutes to eliminate $C^{14}O_2$ not assimilated by the cells. Duplicate 100-μl. portions of the acidified homogenates were plated and the radioactivity determined with a Nuclear-Chicago model D47 gas-flow counter of known efficiency. Self-absorption of β-emission was negligible for the small amounts of solid material plated on each planchet. Chlorophyll *a* determinations were performed on portions of the homogenate, after removal of suspended particles by centrifugation, according to the method of Bruinsma (1961).

Enzyme activity determinations used approximately 200-mg. fresh-weight portions of the L and D cultures. The cells were blotted on moist filter paper and transferred to a glass homogenizer to which were added 2 ml. of 0.01 *M* Tris, pH 7.8, and a small piece of glass-fiber filter paper to facilitate the grinding. Following homogenization for about two minutes at 0° a portion of the homogenate was used for chlorophyll *a* determination, and the rest centrifuged at 0° and 30,000 *g* for 10 minutes. Portions of the supernatant of the centrifuged homogenate were then subjected to enzyme activity analyses as described below.

Most of the assays involved directly, or were coupled to, the oxidation or reduction of the nicotinamide adenine dinucleotides. These assays were carried out in a total volume of 1 ml. in 1-cm. cuvettes. The change in absorbance at 340 mμ was recorded with a Beckman DK ratio-recording spectrophotometer against a distilled water blank. The amount of supernatant from the cell homogenates used as an enzyme source was adjusted to give rates of change or absorbance of 0.1 to 0.5 optical density units per minute. Controls from which substrate was omitted were always run. The methods used for the various enzyme assays have been described previously by Campbell, Hellebust and Watson (1966).

RuDP carboxylase activity was determined from the amount of radioactivity incorporated when the extract was incubated with $NaHC^{14}O_3$ and RuDP, by a modification of the methods of Horecker, Hurwitz and Weissbach (1956). The reaction mixture contained 25 μmoles of Tris (pH 7.8), 0.05 μmoles of $NaHCO_3$, 1 μC. of $NaHC^{14}O_3$, 1.5 μmoles of RuDP, and extract, in a final volume of 250 μliters. After 10 minutes, the reaction was stopped with 25 μliters of 1 N HCl. The mixture was aerated to drive off unreacted CO_2, a 25-μliter sample was plated and dried, and its radioactivity was counted with a Nuclear-Chicago model D47 gas-flow detector.

Five μmoles of R-5-P and 5μmoles of adenosine triphosphate were substituted for RuDP in the RuDP assay system for the measurement of overall activity of R-5-P isomerase, and RuDP carboxylase.

The problem of aliquoting *Acetabularia* into replicate samples for the various types of analyses was solved by using approximately the same number of cells per sample, and then using the chlorophyll content, obtained from determinations of aliquots of homogenates of the samples, as a common denominator.

Abbreviations. R-5-P = ribose-5-phosphate, Ru-5-P = ribulose-5-phosphate, RuDP = ribulose-1.5-diphosphate, FDP = fructose-1.6-diphosphate, P6A = 3-phosphoglyceric acid, 6-3-P = glyceraldehyde-3-phosphate, $NADH_2$ = reduced nicotinamide-adenine dinucleotide, $NADPH_2$ = reduced nicotinamide adenine dinucleotide phosphate.

RESULTS

A first experiment established the relation between light intensity and rate of photoassimilation of CO_2 for cells taken from the middle of the light and dark phases. Figure 1 shows that the photoassimilatory rates of the L sample were considerably higher than those for the D sample at light-limiting as well as at light-saturating intensities. At 500 lux (light-limiting) the rate of the L cells is about 60%, and at 15,000 lux (light-saturating) about 72% higher than the rates for D cells for the corresponding light intensities. Similar data on the relationship

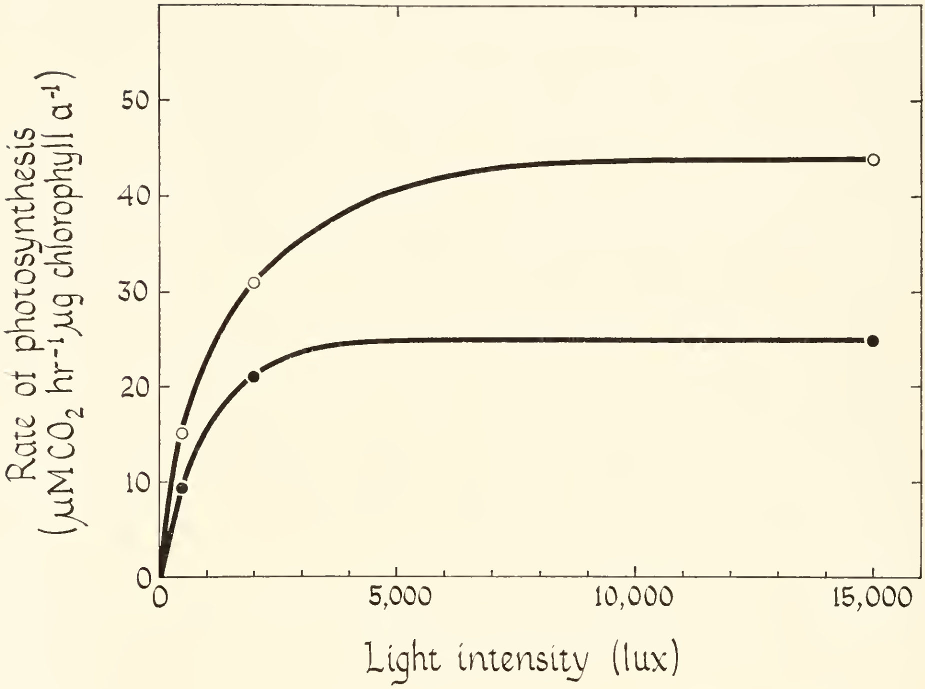

FIGURE 1. Light curve for photoassimilation of CO_2 by *Acetabularia* cells grown on a regime of 8 hours of light at 3000 lux and 16 hours of darkness. The curve with closed circles represents cells harvested at the middle of the dark period, and the curve with open circles represents cells from the middle of the light period.

TABLE I

*Rates of photoassimilation of CO_2 and RuDP carboxylase activities of Acetabularia cells harvested at the middle of the light (L) and the dark (D) period**

	Experiment 1			Experiment 2		
	L	D	L/D	L	D	L/D
Rate of photoassimilation of CO_2 at the following light intensities:						
a) 500 lux	—	—		0.009	0.007	1.3
b) 2500 lux	0.021	0.014	1.5	0.012	0.008	1.5
c) 15,000 lux	0.024	0.017	1.4	0.018	0.009	2.0
RuDP carboxylase activity at the following substrate concentrations:						
a) 2×10^{-2} M HCO_3^-	0.029	0.026	1.1	0.040	0.035	1.1
b) 2×10^{-3} M HCO_3^-	0.009	0.007	1.3	0.012	0.010	1.2

* All the figures represent μmoles CO_2 hr.$^{-1}$ [μg. chlorophyll a]$^{-1}$.

between light intensity and photoassimilation rates for L and D cells are also presented in Tables I, II, and III, and confirm on the whole the relationship presented graphically in Figure 1.

Two experiments correlating the activities of RuDP carboxylase with rates of photoassimilation of CO_2 are recorded in Table I. While the photosynthetic rates of the L cells were consistently higher at all three light intensities than the corresponding rates for D cells, the activities of RuDP carboxylase at two different substrate concentrations were only slightly, and probably not significantly, higher in the L cells than the D cells. Moreover, the observation that RuDP carboxylase from L and D cells responds similarly to changes in substrate concentration indicates that the enzymes from the light and dark cells are kinetically similar. At the higher bicarbonate concentration the activities of RuDP carboxylase are sufficient, or even in excess of those required to account for the corresponding rates of photoassimilation of CO_2 by the intact cells at light saturation (15,000 lux).

A similar experiment, that included assays of several additional enzymes involved in the reductive pentose phosphate cycle, is presented in Table II. Though the photoassimilation rates of L cells were 1.2 to 1.5 times greater than those shown by D cells, the activities of RuDP carboxylase in the extracts appeared to be identical. Similarly, the overall activities of R-5-P isomerase, Ru-5-P kinase, and RuDP carboxylase were also approximately the same for L and D cells. The low activity of this sequence relative to RuDP carboxylase alone was probably due to rate-limiting concentration of the intermediate substrates Ru-5-P and RuDP

TABLE II

*Rates of photoassimilation of CO_2 and activities of enzymes involved in the reductive pentose phosphate cycle of Acetabularia cells harvested at the middle of the light (L) and the dark (D) period**

	L	D	L/D
Rate of photoassimilation of CO_2 at the following light intensities:			
a) 500 lux	0.013	0.009	1.4
b) 2500 lux	0.017	0.014	1.2
c) 15,000 lux	0.022	0.015	1.5
Enzyme activities:			
RuDP carboxylase at			
a) 2×10^{-2} *M* HCO_3^-	0.037	0.037	1.0
b) 2×10^{-3} *M* HCO_3^-	0.010	0.011	0.9
Overall activity of R-5-P isomerase, Ru-5-P kinase, and RuDP carboxylase**	0.020	0.019	1.1
Phosphoglycerate kinase	0.88	0.80	1.1
Glyceraldehyde-3-P dehydrogenase:			
$NADH_2$-dependent	0.14	0.13	1.1
$NADPH_2$-dependent	0.06	0.06	1.0

*All the figures represent μmoles substrate $hr.^{-1}$ [μg. chlorophyll a]$^{-1}$. The substrates used for these calculations were as follows for the different reactions: photoassimilation and RuDP carboxylase, CO_2; aldolase, FDP; phosphoglycerate kinase, PGA; glyceraldehyde-3-P dehydrogenase, $NADH_2$ or $NADPH_2$.

** In the presence of 2×10^{-1} *M* HCO_3^-.

TABLE III

*Rates of photoassimilation of CO_2 and activities of enzymes involved in the reductive pentose phosphate cycle of Acetabularia cells harvested at the middle of the light (L) and the dark (D) period**

	L	D	L/D
Rate of photoassimilation of CO_2 at the following light intensities:			
a) 500 lux	0.006	0.006	1.0
b) 2500 lux	0.014	0.012	1.2
c) 15,000 lux	0.026	0.018	1.4
Enzyme activities:			
RuDP carboxylase*	0.032	0.030	1.1
Phosphoglycerate kinase	0.88	0.92	1.0
Glyceraldehyde-3-P dehydrogenase:			
$NADH_2$-dependent	0.11	0.13	0.8
$NADPH_2$-dependent	0.16	0.16	1.0
Triose isomerase	0.22	0.21	1.0
Aldolase	0.044	0.050	0.9
Transketolase	0.024	0.025	1.0
Transaldolase	0.033	0.030	1.1
R-5-P isomerase	0.22	0.24	0.9
Ru-5-P kinase	0.13	0.14	0.9

* All the figures represent μmoles substrate $hr.^{-1}$ [μg. chlorophyll a$]^{-1}$. The substrates used for these calculations were as follows for the different reactions: photoassimilation and RuDP carboxylase, CO_2; phosphoglycerate kinase, PGA; glyceraldehyde-3-P dehydrogenase, $NADH_2$ or $NAPH_2$; aldolase, FDP; transketolase and transaldolase, $NADH_4$; R-5-P isomerase, R-5-P; Ru-5-P kinase, Ru-5-P, and triose isomerase, G-3-P.

** In the presence of 2×10^{-2} *M* HCO_3^-.

during the relatively short (10-minute) reaction period, rather than to appreciably lower activities of R-5-P isomerase and Ru-5-P kinase relative to that of RuDP carboxylase. The activities of phosphoglycerate kinase, and glyceraldehyde-3-P dehydrogenase, both $NADPH_2$- and $NADH_2$-dependent, were also not significantly different for light and dark cells. The results indicate that the activities of all the enzymes tested were considerably higher than those required to account for the observed rates of photoassimilation of CO_2 at light saturation.

Since theoretically any enzyme required for the operation of the reductive pentose phosphate cycle could be the limiting factor for CO_2 assimilation at light saturation, we performed a final series of experiments that included assays for a total of 9 enzymes in the cycle (Table III). The data show clearly that the activities of none of these enzymes differ to a significant degree in extracts from L and D cells.

The activities of RuDP carboxylase, aldolase, transaldolase, and transketolase were of the same order of magnitude as the rate of photoassimilation of CO_2 at light saturation, while those of phosphoglycerate kinase, $NADH_2$ and $NADPH_2$-dependent glyceraldehyde-3-P dehydrogenase, triose isomerase, R-5-P isomerase, and Ru-5-P kinase were considerably higher than necessary to account for the observed rates of photoassimilation in either L or D cells. The activities of $NADPH_2$- and $NADH_2$-dependent glyceraldehyde-3-P dehydrogenase were of a similar magnitude in this experiment, while the $NADH_2$-dependent activity was approximately twice as great as the $NADPH_2$-dependent activity in the experiment recorded in Table II.

DISCUSSION

The data presented in this paper on photoassimilation of CO_2 by *Acetabularia crenulata* cells demonstrate that the photosynthetic rhythm is expressed at light-limiting as well as light-saturating intensities, although the amplitude of the rhythm is less pronounced at the lower light intensities. These results are in full agreement with those obtained by continuous polarographic oxygen measurements (Terborgh and McLeod, 1967), and strongly suggest that the factor(s) immediately controlling the rate of photosynthesis act at the level of light as well as dark reactions. A recent report by Hoffman and Miller (1966), demonstrating an endogenous rhythm in the Hill-reaction activity of isolated tomato chloroplasts, also suggests that the control of photosynthetic rhythms may involve light reactions. The regulation of a photosynthetic rhythm *via* any of the enzymes of the reductive pentose phosphate cycle would result in changes expressed only at the level of the dark reactions. A second controlling factor would thus have to be assumed to account for the observed rhythm at the light reaction level (quantum yield).

Extracts of *Acetabularia* cells taken from the middle of the light and the dark periods, show no significant difference in the activity and affinity of RuDP carboxylase. This enzyme thus does not appear to be the agent through which the photosynthetic rhythm is expressed as seems to be the case in *Gonyaulax polyedra* (Sweeney, 1965). Dark and light samples, moreover, did not differ significantly in the activities of any of eight other enzymes known to be required for the operation of the reductive pentose phosphate cycle. Five of these enzymes, phosphoglycerate kinase, glyceraldehyde-3-P dehydrogenase, triose isomerase, R-5-P isomerase, and Ru-5-P kinase, had activities considerably above those required to account for the observed rate of photoassimilation of CO_2 at light saturation, and for this reason are not likely to be involved in the control of the photosynthetic rhythm. The activities of RuDP carboxylase, aldolase, transketolase and transaldolase were of the order of magnitude required to account for the observed rates of photoassimilation; however, as already stated, no rhythm was expressed in the activities of these enzymes. All the measurements of enzyme activities were performed on all extracts and employed "optimal" assay conditions; *i.e.,* optimal with respect to pH, substrate concentration, redox conditions, metal ion concentrations, etc. Our results can only be taken to indicate that there is no rhythmic variation in the amount of the enzymes investigated, and in the case of RuDP carboxylase in its affinity for CO_2 (*in vitro*). On the other hand, it is entirely possible that the *activity* of any one of these enzymes could vary in a rhythmic fashion in the cell in response to rhythmic changes in the cellular environment, such as pH, redox potential, ion concentrations or substrate concentration, and therefore, in fact, be involved in the expression of the observed rhythm of photoassimilation of CO_2. Investigation of the possibility of such an *in vivo* rhythm of enzyme activity would necessarily involve measurements of relative turnover rates of pools of intermediate substrates. Unfortunately, experiments of this kind with marine algae present great technical difficulties because of their high salt contents.

Driessche (1966) has recently reported a circadian rhythm in chloroplast shape in *Acetabularia mediterranea.* In the middle of the light period, coincident with maximum rates of O_2 evolution, the chloroplasts become elongate. During the dark period, when O_2 evolution was at a minimum, the shape of the chloroplasts was more spherical. Thus, we now have three rhythms concerning photosynthesis or the chloroplasts of *Acetabularia* to account for: in photosynthetic dark reactions, photosynthetic light reactions (quantum yield), and chloroplast shape. Possibly the endogenous time-keeping mechanism of *Acetabularia* regulates only a single key photosynthetic reaction which in turn underlies two or more manifest oscillations. Such a conservative mechanism could account for parallel changes in photosynthetic activity and maximum capacity if the critical reaction were to moderate the activity or amounts of primary reductant or of the long-wave-length form of chlorophyll *a.* A certain amount of circumstantial evidence stands in accord with possibilities of this kind. Kok (1956) and later Kok, Glassner and Rurainski (1965) have shown with *Chlorella* and spinach chloroplasts that during reversible inhibition of photosynthesis by high light intensities the quantum yield was affected to exactly the same extent as the photosynthetic dark reactions. Kok (1956) has postulated a reversible inactivation of photosynthetic units that also affects the rate of dark reactions. Under quite a contrary set of conditions we found a concurrent deterioration of light and dark reactions in the green flagellate, *Dunaliella tertiolecta,* in response to prolonged darkness or to very dim light (Hellebust and Terborgh, 1967).

Since a relationship has been established between photosynthetic phosphorylation and the structural state of fragments of spinach chloroplasts (Packer, 1962), as well as light-dependent volume changes in chloroplasts *in vitro* (Packer *et al.,* 1965), it may be that the rhythms in photosynthesis and chloroplast shape have a common basis.

In conclusion our evidence indicates that the circadian oscillation in the activity and maximum capacity of photosynthesis in *Acetabularia* thus stands in contradistinction with *Gonyaulax* in which a rhythm in RuDP carboxylase activity may be sufficient to explain the observed rhythm in photosynthetic capacity. The apparent disparity in the control mechanisms of these two organisms can be rationalized from an evolutionary point of view if one makes the assumption that circadian rhythms, where found, have selective value. For plants of diverse ecological proclivities, having photosynthetic rhythms optimal adaptation might entail a fluctuation of maximum capacity as in *Gonyaulax*, in both activity and capacity as in *Acetabularia,* or in activity alone, a case for which we have no clear example at present.

SUMMARY

1. The photosynthetic rhythm of *Acetabularia crenulata* affects both light (quantum yield) and dark reactions in a parallel manner.

2. No significant difference was found between the activity of RuDP carboxylase in the extracts of samples taken at the middle of the light and dark periods nor was any difference detected in the affinity of this enzyme to CO_2. The activity of RuDP carboxylase in the cell extracts was sufficient to account for the observed rates of photoassimilation of CO_2 at saturating light intensities.

3. The activities of eight other enzymes of the reductive pentose phosphate cycle were also shown not to differ to a significant extent in extracts of cells taken at the middle of the light and dark periods. Five of these enzymes (phosphoglycerate kinase, glyceraldehyde-3-P dehydrogenase, triose isomerase, R-5-P isomerase, and Ru-5-P kinase) had activities considerably above those required for the observed light-saturated rate of CO_2 assimilation while the activities of aldolase, transketolase and transaldolase were of the same order of magnitude as the observed rates of photoassimilation.

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CENTRAL NERVOUS SYSTEM CONTROL OF CIRCADIAN RHYTHMICITY IN THE COCKROACH. I. ROLE OF THE PARS INTERCEREBRALIS

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Circadian rhythmicity is now well-established as a common feature of physiological systems of all levels of organization, from intact multicellular organisms to isolated organs, single (protistan) cells and individual enzyme systems. A principal aim of research at both the uni- and multi-cellular levels is to locate and characterize the nature of the self-sustaining oscillation (in control mechanisms) which causes the observed rhythmicity. In multicellular systems the first step must be to inquire whether or not a distinct group of cells functions as a pacemaker, or driver, for the rest of the system. In this series of papers we pursue the many published suggestions that some part (or parts) of the insect brain functions as that pacemaker.

The starting observation concerns decapitated insects; they may survive and move for days or weeks, but their locomotory activity loses its previous circadian rhythmicity (Eidman, 1956; Harker, 1956; Nishiitsutsuji-Uwo, unpublished data). Of the several organs in the head the corpora cardiaca and/or allata appear to have no effect on circadian locomotory rhythms (Eidman, 1956; Fingerman, Lako and Lowe, 1958; Roberts, 1966; Nishiitsutsuji-Uwo, unpublished data). On the other hand there is ample evidence that these rhythms are effected, directly or indirectly, by the brain. For instance, Dupont-Raabe (1957) and Mothes (1960) have shown that the brain exerts an endocrine control over the daily cycle of color change in *Carabus morosus*. Klug (1958) has reported a daily cycle in the number of neurosecretory cells in the brain of *Carabus nemoralis* containing secretory granules (and an associated cycle of nuclear volume change in the cells of the corpus allatum). Rensing (1964, 1966) has reported similar observations on *Drosophila melanogaster*. The suggestion arising from these latter facts is that a rhythmicity of neurosecretion from the pars intercerebralis may be underlying the locomotory rhythm; and this is certainly encouraged by the fact that extracts from corpora cardiaca, which are storage organs for this secretion (Scharrer, 1952), apparently affect the spontaneous electrical activity of isolated nerve cords (*Periplaneta*) *in vitro* (Özbas and Hodgson, 1958). Indeed Eidman (1956) has already concluded from ablation experiments that the pars intercerebralis is involved in the control of circadian rhythms of locomotion in *Carausius*, and Roberts (1966) drew a similar conclusion after making midsagittal bisections of the *Periplaneta* protocerebrum. But neither of these published observations is as yet fully compelling. Eidman's observations, for example, were limited to one day following the surgery;

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in our experience arrhythmicity is an *immediate* and transient consequence of almost any surgery of the brain.

Ingenious experiments by Lees (1964) provide conclusive demonstration that photoperiodic receptors of the aphid *Megoura viciae* are located in the protocerebrum. This protocerebral center is active even after the compound eyes have been blinded, suggesting that the neurosecretory cells in the pars intercerebralis may be implicated both as receptors and as humoral effectors.

If one regards (Bünning, 1936; Pittendrigh and Minis, 1964) photoperiodic induction as an aspect of the entrainment of circadian oscillations by light, he will be further encouraged by Lees' findings to focus attention on the pars intercerebralis as a potential pacemaker for the circadian system in insects.

Harker (1956, 1960a, 1960b) has published well-known and very important conclusions that the suboesophageal ganglion in *Periplaneta americana* is directly responsible for the circadian rhythm of locomotion in that insect. No confirmation of her finding has, however, yet been published, and Roberts (1966) describes repeated failures to obtain Harker's results from apparently identical procedures. In the meantime we conclude that the role of that ganglion remains to be fully established. In any case, Harker's work is of great importance in a quite different respect; it exemplifies the only experimental procedure which yields, in principle, unequivocal evidence of having localized the pacemaking oscillation in the system. Thus she reported transfer of the rhythm's *phase* when she implanted a ganglion into a headless host. Loss of rhythmicity following ablation of some organ is, of itself, equivocal; that organ could be indispensable to the expression of an *assayable* rhythm but in fact be only peripheral to a driving pacemaker left after the ablation, and unable to express its oscillation in terms of the assayed parameter. Indeed restoration of the rhythm by replacing the ablated part is also equivocal unless it includes (as Harker reports for *Periplaneta*) introduction with the implant of a *specific phase* different from that previously expressed by the host.

Roberts' failure to confirm the role of the suboesophageal ganglion, and the other indications noted above that the pars intercerebralis is involved have prompted our own attention, in this series of papers, to the protocerebrum of the cockroach, and to the pars intercerebralis in particular. The dependence of pars intercerebralis neurosecretion on transport by intact axons has precluded our exploitation of the technique of replacing ablated parts, and our conclusions are necessarily limited in this respect.

Materials and Methods

Two species of cockroach employed in these experiments, *Periplaneta americana* and *Leucophaea maderae,* were maintained in temperature-controlled (25° C. ± ½° C.) rooms in cycles of alternating light and dark (LD). Prior to experimental manipulation the animals were placed in monitored activity wheels (Roberts, 1960, 1962) in light-tight constant-temperature (25° C. ± ½° C.) cages equipped with a clock-controlled, water-jacketed 4W cool white fluorescent light source. They were exposed to either regularly alternating light and dark (LD), constant dark (DD), or a combination of these to test their activity rhythms. Only newly emerged adult male roaches were used. Roaches that failed to demonstrate clear rhythmicity, as judged by either a lack or low level of locomotion, or by lack of a clear relationship between the phases of the activity rhythm (determined by the time of the onset of activity) with respect to the phase of the "entraining" LD cycle (generally 12 hours light and 12 hours dark per 24 hours, LD *12*:12) were discarded.

Methods for recording and analyzing activity data are in general use and have been frequently described (Roberts, 1960).

Surgical procedures involved anaesthetizing the roach in its activity wheel by exposing it to CO_2. The animal was then removed from the wheel and its head was wedged and firmly held in a V-shaped opening in a (3″ × 5″ × 1″) plastic box. With its head projecting out of the opening, the animal was taped across the thorax to the bottom of the box and the antennae were strapped to the top surface of the box. The box was then attached to a movable operating platform which was mounted through a universal ball-joint to a mechanical stage. The bottom of the box was equipped with an inlet for CO_2 supplied from a cylinder through a line connected to a heat exchange-coil immersed in a constant temperature (25° C. ± 0.1° C.) water bath. The head of the roach was illuminated from above on either side by focused microscope lamps equipped with heat absorbing filters. This combination of heat filters and the stream of temperature-controlled CO_2 passing over the body and around the head of the roach allowed the animal to be anesthetized and immobilized for long periods without subjecting it to temperature pulses.

Prior to cutting, the entire head was cleaned with 70% ethanol. Cutting tools, consisting of micro-scalpels made from razor blade fragments held in blade holders, and finely sharpened forceps, were sterilized in 70% ethanol. The animal was positioned with the frontal portion of the head facing the operator in such a way as to allow comfortable access to and visibility of this region through a binocular microscope.

The first step in the procedure involved exposing the protocerebrum by cutting and removing a window of cuticle directly above this portion of the brain. The piece of cuticle was carefully placed on a sterile surface and saved for later resealing of the wound with dental wax. Once the cuticle square was removed and the tracheae and fat body tissues were cleaned away, the protocerebral lobes were clearly visible and seen bathed in "blood" which was removed just prior to surgery with a sterile cotton swab (see Fig. 1). A cut was made on each side of the protocerebral lobes just lateral to the mid-sagittal point such that a wedge containing the pars intercerebralis was freed from the remainder of the brain and could be removed with forceps. At this point notation was made by the operator grading the depth of the cuts as either complete or incomplete. A complete cut was one which, in the operator's judgment, contained sufficient of the underlying brain tissue in the pars intercerebralis, in addition to the mid-lateral portions of the protocerebral lobes, to remove all neurosecretory cells present in those areas. Incomplete cuts were those which did not penetrate deeply enough to remove these areas. In no cases reported here were the cuts sufficiently deep to result in mid-sagittal bisection.

In the course of these experiments many attempts were made to burn out the pars intercerebralis with an electric micro-needle. However, any needle used was not satisfactory to destroy the pars intercerebralis only. When the pars intercerebralis was completely burned, serious damage was inflicted on a wide adjacent

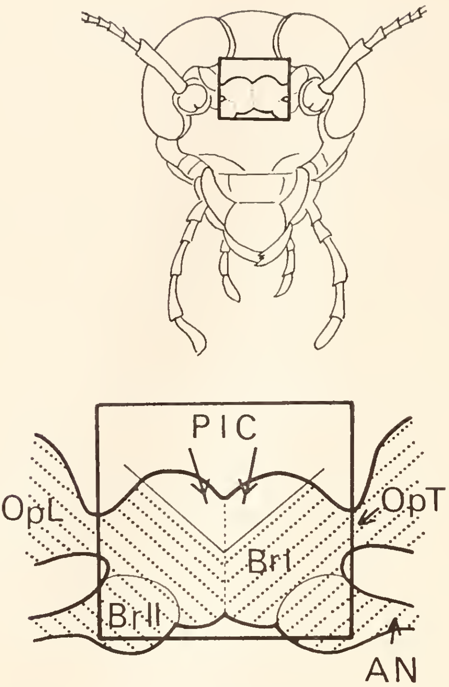

FIGURE 1. (a) A diagrammatic representation of the head of a cockroach showing the $\sim$ 1.5-mm. square "window" made by removing a piece of cuticle from the front directly above the protocerebrum (Br I), thus allowing access to the pars intercerebralis (PIC). (b) Frontal view of the brain under the window illustrating the removal of the part of the brain containing the pars intercerebralis (white area). PIC: pars intercerebralis, Br I: protocerebrum, Br II: deutocerebrum, OpL: optic lobe, OpT: optic tract, AN: antennal nerve.

area. Since the brain is a soft, yielding structure, surgical ablation of this area was also not completely satisfactory. However, the cockroaches survived for long periods post-operatively and the extent of surgery could be easily checked by histological methods. Usually the first cut and removal of one side of the pars intercerebralis were easier than the second cut and removal of the other side. The areas immediately beneath the indentation of the pars intercerebralis, where the medial neurosecretory cells are located, were especially difficult to remove without damaging the area where the nerve tracts of the medial neurosecretory cells cross. Although notation of "complete" or "incomplete" ablation was made by the operator, histological confirmation was required, especially in those cockroaches which re-established a normal rhythmic pattern post-operatively. Histology of the brain of cockroaches showing arrhythmicity after surgery was also important. Since some operated cockroaches remained arrhythmic for 3 weeks before they resumed normal rhythmicity, long-term observation was required to establish clearly post-operative arrhythmicity. Therefore, most of the arrhythmic animals were kept until they died. After the effect of surgery on the rhythm had been assayed by recording the animal's activity for many days, the brains of most animals, especially those in which normal rhythms reappeared post-operatively, were fixed with Helley's fixative.

Halmi's (1952) aldehyde fuchsin-azan method as modified by Scharrer (personal communication) was used for the histological demonstration of neurosecretory materials.

Examination of the sections containing portions of the pars intercerebralis for the presence of neurosecretory cells is not difficult in brains of unoperated animals as one can use the "crotch" of the protocerebral lobes as a marker which, when present in the section, indicates to the observer that the section probably contains portions of the pars intercerebralis. Sections containing this "crotch," which also pass through the optic tracts and lobes, are the ones generally found to contain the brightly stained neurosecretory cells. In preparations where this "crotch" is absent or changed in shape because of surgical removal of the pars intercerebralis, precise serial orientation of the sections becomes a much more difficult problem. Despite these problems, there is no question about the identification of neurosecretory cells in well-stained sections when they are present. For obvious reasons, the total *absence* of neurosecretory cells in operated animals is much more difficult to discern. Control sections consisting of brains from unoperated animals were run parallel with each experimental group material in order to verify the success of the staining procedure.

TABLE I

Effect of ablation of the pars intercerebralis on the circadian locomotory rhythm in 45 operated cockroaches. Operation Grade; see text. Activity Level: Lower or Higher as compared to pre-operative normal level

Group	I	II
Post-operative rhythm	No rhythm	Rhythm
No. of animals	19*	28*
Operation grade		
Incomplete	4	9
Complete	13	10
No grade	2	9
Days of post-operative observation	35(14–78)	29(10–60)
Days until post-operative appearance of rhythm	—	9(0–24)
Activity level		
Normal	6	12
Lower	0	1
Higher	13	9
Lower then higher	0	6
Neurosecretory cells		
Present	—	11
Absent	3	8
Questionable	—	3

* Of 19 animals in Group I, 3 animals showed a questionable activity pattern and of 28 animals in Group II, 13 animals showed a rhythm but not quite a normal rhythm (see text).

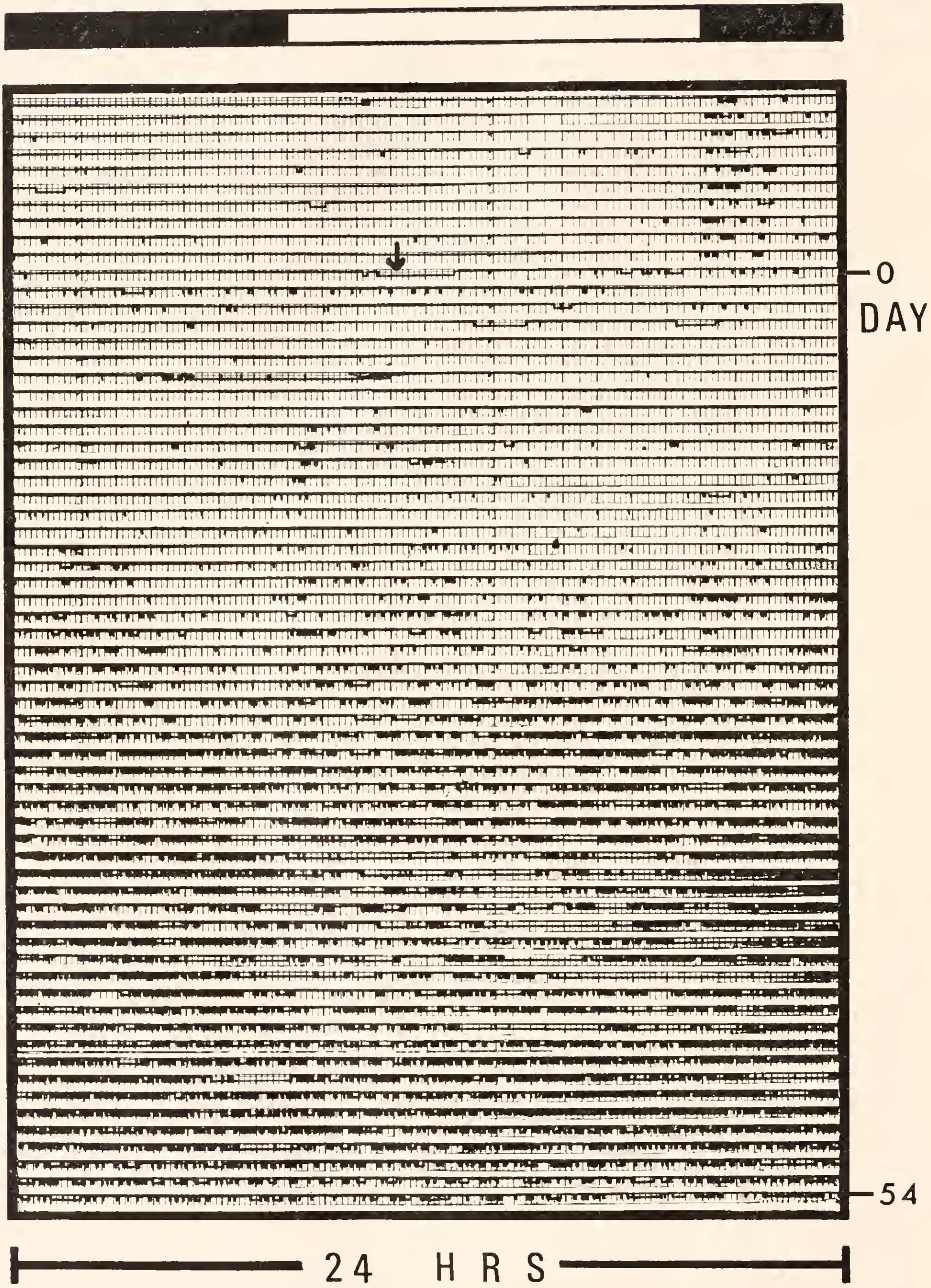

FIGURE 2.

RESULTS

I. Effect of the removal of the pars intercerebralis

The data summarized in Table I show that a total of 47 operations on the pars intercerebralis was performed. Of these, 19 animals displayed no rhythmicity of activity post-operatively (Group I) while 28 displayed activity rhythms following removery from the operation (Group II).

1. Group I. No rhythm post-operatively (19 animals).

All the animals in Group I showed arrhythmic activity patterns following surgery (Table I). Of these, 4 were sacrificed or died within 3 weeks after surgery, which was considered the minimum observation period required to establish the absence of an activity rhythm. The average observation period for all animals in this group was 35 days, with a range of from 14 to 78 days. Only 3 arrhythmic animals served for histological examination and no neurosecretory cells were found. Thirteen out of the 19 of these operations were rated as "complete" at surgery, and 4 were rated as "incomplete."

The majority of animals in this arrhythmic group displayed abnormally high levels of post-operative activity as compared to pre-operative levels (see Fig. 2).

2. Group II. Rhythm post-operatively (28 animals)

Ten of the 28 animals in this group were rated as having "complete" removal of the pars intercerebralis at surgery; 9 were rated "incomplete"; 9 were not rated. In all of them the activity data generally showed a low level of locomotory activity for a few days after the operation, followed by a return to the normal level or to a sudden higher level. This transient post-operative suppression of activity—and hence of apparent rhythmicity—establishes clear demands on the duration of post-operative observation before reliable conclusions can be reached concerning the role of the tissue removed in the operation. In 9 cases activity was higher, in 6 cases first lower then higher, and in 1 case lower post-operatively than pre-operatively. The activity pattern became overtly rhythmic (except in 5 cases which showed unclear onsets of activity) at varying times following surgery within 2 weeks (0–15 days) (see Figs. 3, 4), but in 3 cases activity remained arrhythmic for about 3 weeks (19, 20 and 24 days), after which the rhythm became apparent. The rhythms that eventually developed in this group show atypical features in 4 respects.

First, of four animals showing an LD rhythm that were subsequently placed in DD, only 3 showed a clear circadian rhythm (Fig. 4); one of them became immediately aperiodic as though its former periodicity had been entirely imposed by the light cycle.

Second, in 5 animals the activity rhythm which developed showed clear signs of a *bimodality* which is rarely seen in normal animals (Fig. 4). A distinct peak

FIGURE 2. Loss of the activity rhythm in *Leucophaea maderae* following complete ablation of the pars intercerebralis (Group I). The operation was performed on day 0 (time is indicated by arrow). Usually, animals showed high activity for 1–2 days after operation, then moderate or low activity appeared for a period (about 1 week). After this, activity increased to a very high level without showing any rhythm for 1–2 months; it decreased for a week before the animal's death.

This animal was sacrificed on day 55 and histological sections of its brain showed no neurosecretory cells. The light-dark regime is indicated at the top of the figure (open bar = light, solid bar = dark).

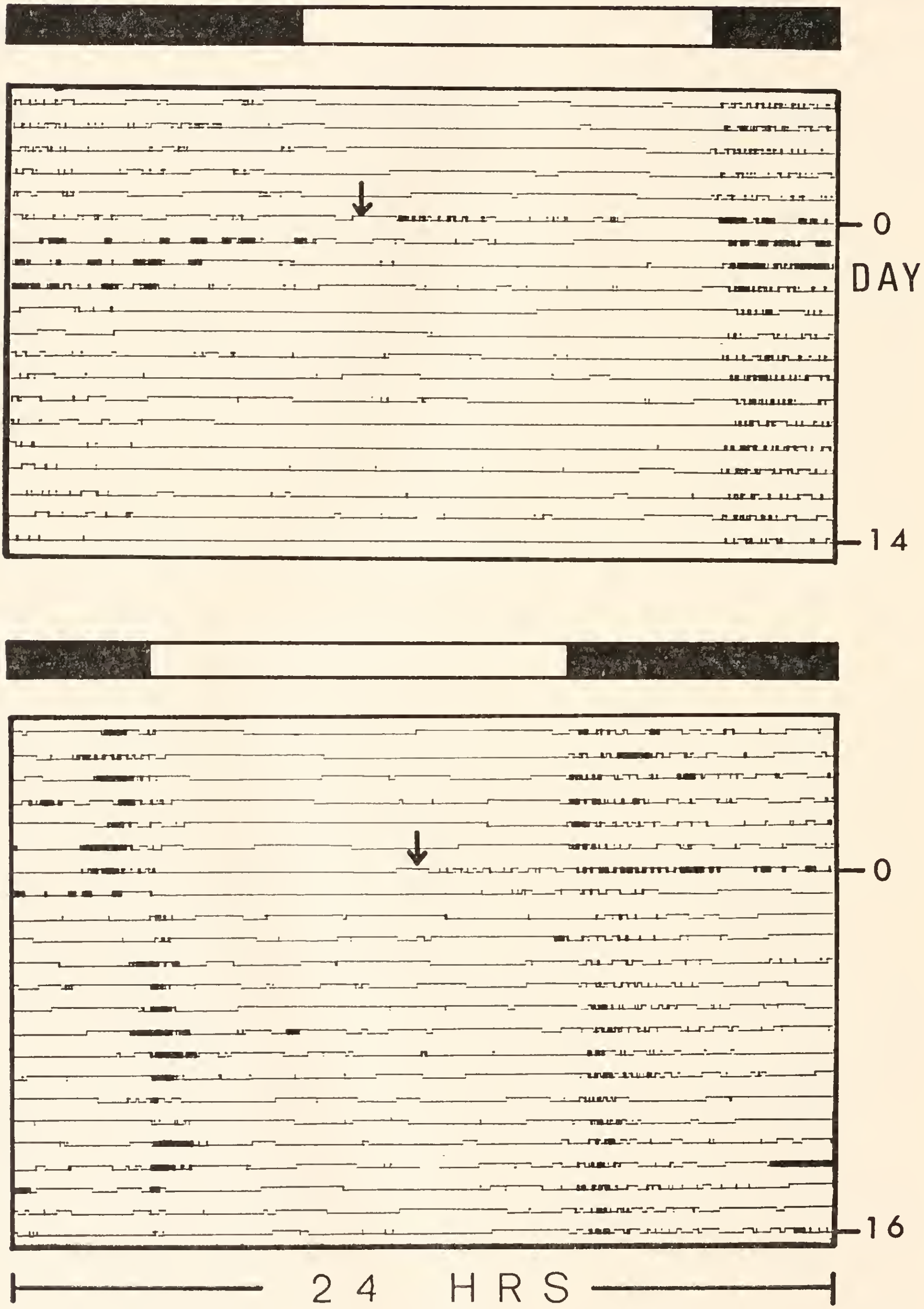

Figure 3.

of activity immediately *follows* dawn. Figure 3b shows one of the rare cases of bimodality in a normal animal; after operation its dawn peak switched from anticipating dawn to following dawn.

Third, as seen in Figure 5a, the operation caused the activity period, which is normally restricted to darkness, to extend as a single peak far into the end of the dark period.

Fourth, one animal remained unimodal but its activity maximum switched from the dark to the light part of the entraining LD cycle (Fig. 5b).

Most animals were sacrificed after the normal rhythm of locomotion was established post-operatively, in order to examine the brain histologically. The brains appeared normal except for a flat angle of indentation ("crotch") of the protocerebrum. In general, cut surfaces of the brain were covered with a neural lamella and perilemma which, although continuous with the uncut surface, were only about $\frac{1}{3}$ or $\frac{1}{2}$ the thickness of the intact layers.

Eleven of the 22 post-operative rhythmic animals which served for histological observation (Table I) revealed the presence of neurosecretory cells, many of which were filled with neurosecretory material. In 3 of 11 cases, only lateral neurosecretory cells of one side of the protocerebrum were found. An additional 3 cases showed questionably positive evidence, *i.e.,* very weakly stained cells, and in 8 cases, no neurosecretory cells were found.

II. Effect of actinomycin D on the pars intercerebralis

Since the brain hormone produced in the neurosecretory cells of the pars intercerebralis has been shown to be a protein-like substance (Ichikawa and Ishizaki, 1963), chemicals which block such synthesis, such as actinomycin D, might be expected to have an effect on the circadian rhythm of locomotion if this portion of the brain does indeed function as a humoral effector in controlling activity. We attempted to block synthesis in the pars intercerebralis using chips of gel containing actinomycin D. These chips were inserted from the top surface of the protocerebrum by making a small cut in the brain sheath and forcing the chips into the mid portion of the pars intercerebralis bilaterally.

The gel was prepared in the following way: 1 mg. actinomycin D was placed in a pre-heated (40° C.) petri dish (5 cm. diameter) to which were added 2 or 3 drops of acetone and then several drops of 2% Bacto agar solution (40° C.). This mixture was left at room temperature in the dark until the acetone and water had completely evaporated. The resulting dried disc of agar gel was cut into small pieces of $0.005 \sim 0.014$ mm.^{2} which contained approximately $0.02 \sim 0.05$ μg. of

FIGURE 3. Persistence of the activity rhythm in *Periplaneta americana* following the operation (Group II). The operation was performed on day 0 (time indicated by arrow) and the rhythm persisted in LD to day 14 (a) and day 16 (b). The animals were sacrificed on day 15 (a) and 17 (b), respectively, and histological sections of their brains showed the presence of neurosecretory cells. The light-dark regime is indicated at the top of the figures (open bar = light, solid bar = dark). (a) Typical example of persistence of the activity rhythm. (b) Biphasic rhythm: The activity rhythm which developed showed clear signs of a bimodality. This animal is one of the rare cases of bimodality in a normal animal (see pre-operative record); after operation its dawn peak switched from anticipating dawn to following dawn.

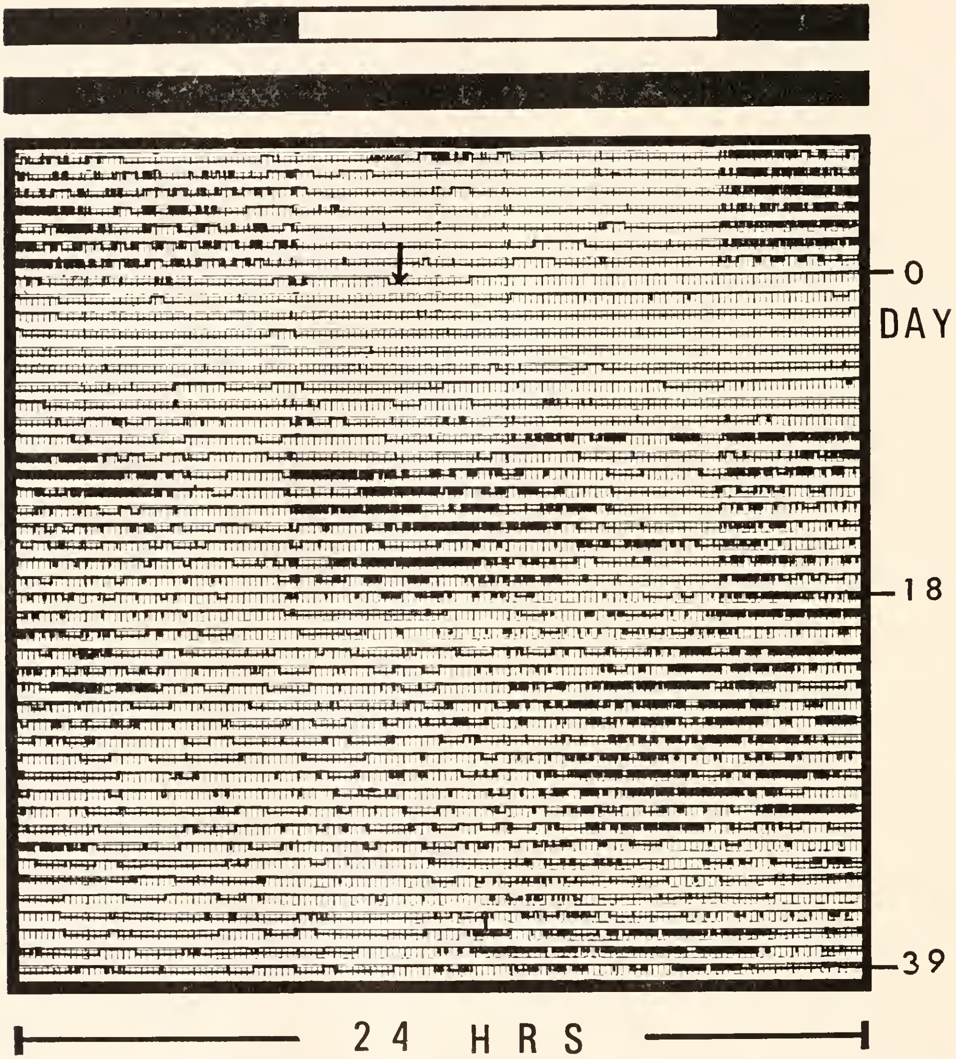

FIGURE 4. Biphasic rhythm in *Leucophaea maderae* following recovery from the operation. Within a week after the operation, the animal showed very low levels of activity; then the activity level suddenly increased and a biphasic rhythm, with the onsets of activity clearly phased to dawn and dusk, appeared. From days 19–39, the animal showed a free-running rhythm in DD. The animal was sacrified for histology on day 40 and neurosecretory cells were found.

actinomycin D. Control chips were made in the same way using eosin instead of actinomycin D.

As seen in Table II, 7 animals which received actinomycin D gel were observed for 35 days (21 ∼ 57 days) post-operatively. Activity levels were generally normal or lower post-operatively than pre-operatively. None of these animals recovered

TABLE II

Effect of exposing the pars intercerebralis to actinomycin D on the circadian locomotory rhythm in cockroaches

Experimental procedure	No. of animals	Post-operative observations			
		Days of observation	Activity level	Rhythm	Days until appearance of rhythm
Act. D → PIC	7	35(21–57)	Normal 4 Other 3	None	—
Eosin → PIC	5	41(13–61)	Normal 4 Other 1	Normal	3(1–7)
Act. D → 5AG	2	43, 48	Normal	Normal	0*

Act. D. = Actinomycin D gel; PIC = Pars intercerebralis; 5AG = 5th abdominal ganglion.
* 0 means immediately after implantation.

locomotory rhythm after operation. Four out of 7 operated animals were dead at the end of the observation period (average days of survival was 38 days) and the others were sacrificed for further observation of the brain. Usually, the protocerebrum was partially (upper half or pars intercerebralis only) histolyzed and there was always a large pigmented tumor-like mass in the place of degenerated brain tissue. The optic lobes and tracts appeared to be intact. Histological preparations were unfortunately not made.

On the other hand, eosin gel-implantation did not affect activity or locomotory rhythms except that temporarily low arrhythmic activity persisted for 2 to 6 days post-operatively. All animals (5) survived until they were sacrificed for observation of the brain which showed no changes in shape nor pigmentation. Histological observations confirmed that the brains were normal except that some parts of the pars intercerebralis had been damaged. None of the implanted gel could be detected.

As an additional control, an actinomycin D gel chip (2 × the usual size) was inserted into the 5th abdominal ganglion in 2 animals. Both showed normal level of activity and rhythms immediately after the implantation.

From these experiments it was evident that although the implantation of the gel caused some physical damage to the pars intercerebralis, normal locomotory rhythmicity resumed shortly after the operation unless the gel contained actinomycin D. We conclude that actinomycin D was responsible for loss of the rhythm. But it is equally clear that our original goal—the local blockage of m-RNA synthesis—was not attained; the amounts of actinomycin D in the chips were too great and the positive results we obtained reflected an extensive "chemical surgery" of the dorsal section of the protocerebrum.

Why the tumor-like change happened after implantation of actinomycin D is a puzzle. Although there is a report that subcutaneous administration of actinomycin S and L to various stocks and strains of mice produced sarcomas (Kawamata, Nakabayashi, Kawai, Fujita, Imanishi and Ikegami, 1959), there is as yet no information on their effects in invertebrate tissues.

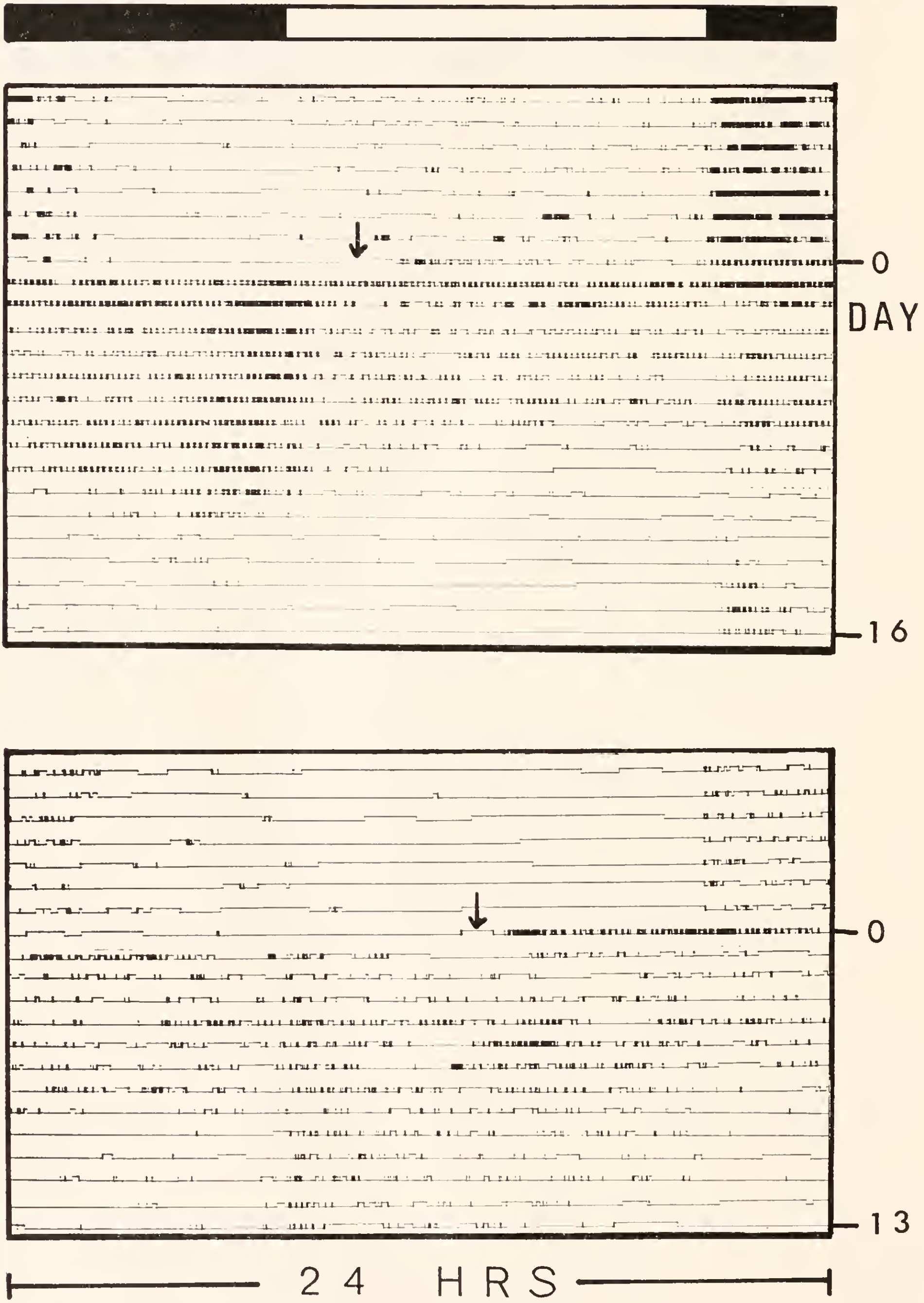

FIGURE 5.

Discussion

The data presented here are interpreted as evidence demonstrating a relationship between the neurosecretory cells of the pars intercerebralis and the circadian locomotory rhythm in the cockroach. Removal of this portion of the brain together with the neurosecretory cells results in a loss of rhythmicity provided that all neurosecretory cells, including the lateral and medial groups, are removed or incapacitated during surgery. In over one-half of those cases studied in which rhythmicity "regenerated" post-operatively, neurosecretory cells have been demonstrated to be present, and most of them were presumably functioning (as judged by the presence of neurosecretory material in the cells). As noted earlier, failure to demonstrate neurosecretory cells by the presence of stained neurosecretory substance is not a clear demonstration that they were not present in the specimen; it merely means they could not be found or were not functional. Thus, in light of this, the 11 positive cases and the 3 questionable cases out of 22 brains studied are even stronger evidence than the figures alone would suggest of a correlation between the presence of neurosecretory cells in the pars intercerebralis and the persistence or reappearance of rhythmic locomotory patterns in operated animals. This, together with the demonstration of a permanent loss of rhythmic activity in 19 of the 47 animals studied, further points up the important relationship between the pars intercerebralis and circadian rhythms of locomotion in roaches.

The nature of this relationship remains unclear; however, evidence presented here is suggestive of a system in which the neurosecretory material elaborated by the neurosecretory cells of the pars intercerebralis, in some way acts as a suppressor of locomotory activity. Thus, surgical removal or incapacitation of the productive sites or routes of dispersal of this material "releases" the animal from the inhibitory control of the brain, and its general level of activity is raised. This would explain the relatively higher level of activity post-operatively in animals rendered arrhythmic as compared to those in which rhythms "regenerated," since in the former the sites producing the locomotory suppressor have been removed or incapacitated whereas in the latter they have been only temporarily inactivated or partly removed. This interpretation is compatible with the evidence of Özbas and Hodgson (1958) who demonstrated that extracts of the corpora cardiaca believed to be a storage organ of brain neurosecretory material (Scharrer, 1952) of the cockroach cause a decrease in the spontaneous electrical activity of isolated ventral nerve cords *in vitro*.

To explain the loss of rhythmicity resulting from the surgical removal of these neurosecretory cells would presumably require that the neurosecretory material is either rhythmically produced, stored or released. The evidence of Klug (1958), demonstrating a correlation between the activity cycle of *Carabus nemoralis* M. with changes in the volume of the nuclei of the cells of the corpora allata and with changes in the number of neurosecretory cells in the brain which contain secretory granules, together with reports of similar findings in *Drosophila* (Rensing,

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FIGURE 5. Abnormal activity rhythms in *Periplaneta americana* following removal of the pars intercerebralis (Group II). The operation was performed on day 0. The animals were sacrificed on day 17 (a) and 14 (b), respectively, and no neurosecretory cells were found in their brains. (a) The operation caused the activity period to extend as a single peak far into the end of the dark period. (b) This animal remained unimodal but its activity maximum switched from the dark to the light part of the entraining LD cycle.

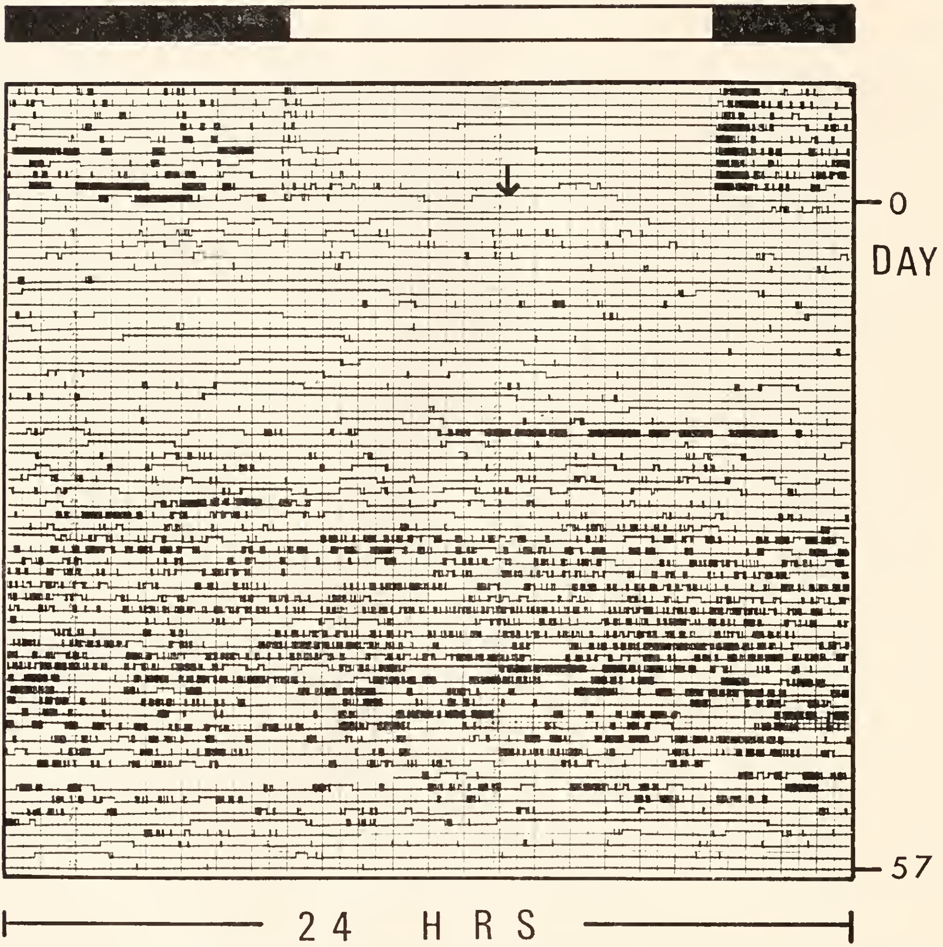

FIGURE 6. Effect of actinomycin D on the pars intercerebralis of *Leucophaea maderae*. Actinomycin D gel was inserted bilaterally into the pars intercerebralis on day 0. The animal showed low activity for 19 days after the operation, then activity increased to a very high level without showing any rhythm for 5 weeks. This activity pattern is similar to that of animals in which the pars intercerebralis was ablated (*cf.* Fig. 2). The animal died on the 57th day following gel insertion.

1964, 1966), suggests that rhythmic neurosecretion may be the mechanism operating in the cockroach. This mechanism is also favored on the basis of evidence in which no role could be demonstrated for the corpora allata and corpora cardiaca in the locomotory rhythm of cockroaches (Roberts, 1966), *Carausius* (Eidman, 1956) and *Romalea* (Fingerman *et al.*, 1958).

Roberts (1966) has concluded that a relationship exists between the neurosecretory cells of the pars intercerebralis and the locomotory rhythm in the cockroach on the basis of his midsagittal bisection through the pars intercerebralis, which caused a loss of the locomotory rhythm. Midsagittal bisection between the

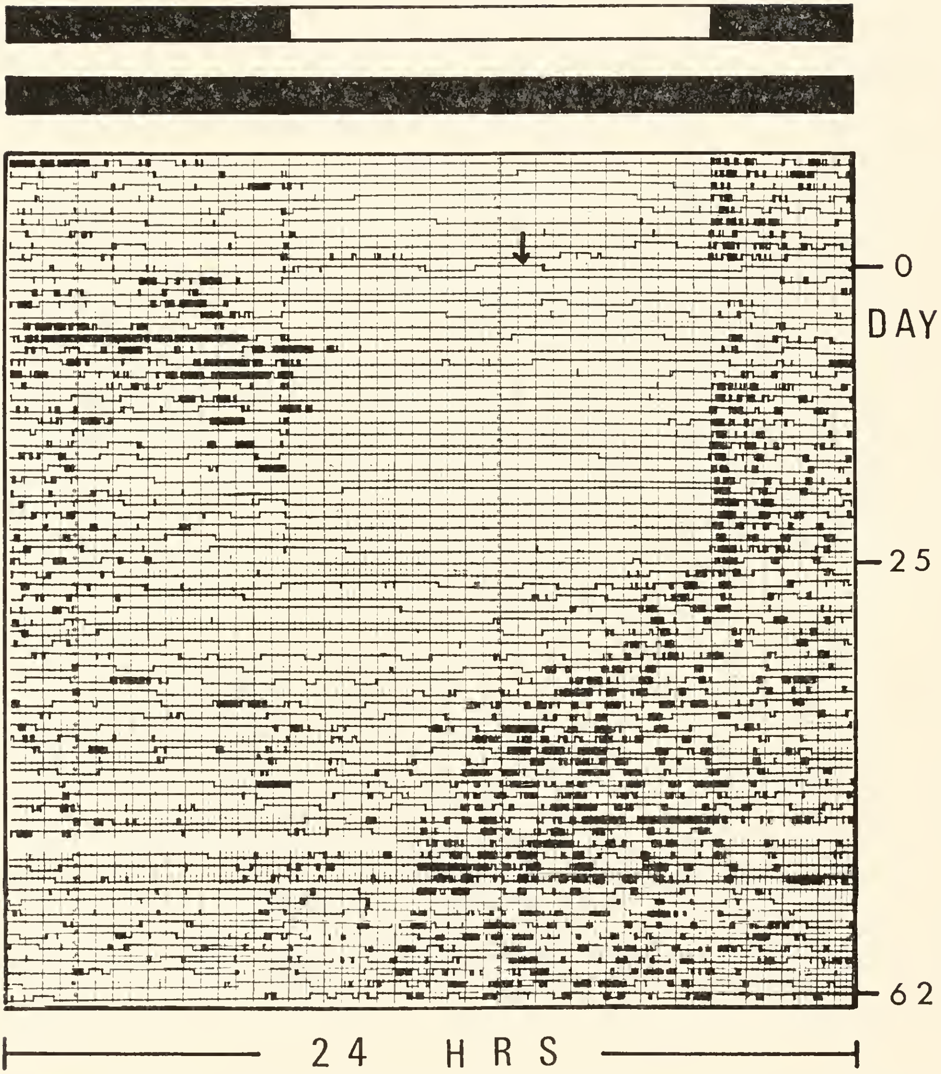

FIGURE 7. Effect of eosin control gel on the pars intercerebralis of *Leucophaea maderae.* Gel containing eosin instead of actinomycin D was inserted bilaterally into the pars intercerebralis on day 0. Within a week after the operation, the animal resumed its pre-operative rhythm and showed a free-running rhythm in DD (on day 25–62). Histological observation confirmed (on day 68) that the brain was normal except that some parts of the pars intercerebralis had been damaged and that none of the implanted gel could be detected.

two lobes of the protocerebrum severs the nerve tracts which emanate from the medial neurosecretory cells at the point where they cross to opposite sides. We have found that in post-operative animals in which only some lateral neurosecretory cells remained intact, normal rhythmicity could be re-established post-operatively.

Thus, Roberts' (1966) demonstration of post-operative arrhythmicity resulting from midsagittal bisection does not, of itself, constitute proof of a relationship between the circadian rhythmicity of locomotion and the neurosecretory cells of the brain, since the midsagittal bisection does not sever the nerve tracts emanating from the lateral neurosecretory cells. These tracts presumably remain intact and functioning following this operation. Furthermore, most of our operated animals showed normal locomotor rhythms following midsagittal bisection of the brain (unpublished data). Roberts, also, has shown normal rhythmicity in some cases. He has emphasized only "arrhythmicity" following midsagittal bisection of the brain. Results on the effects of various nerve sectionings, including midsagittal bisection, on the circadian locomotory rhythm will be reported in the near future.

There is a suggestion from the data in Table I that there may be an additional explanation for the reappearance of locomotory rhythmicity in the operated animals. In the majority of animals, rhythms appeared between 0 and 15 days and it has been suggested that this resulted from an incomplete removal of the neurosecretory cells of the pars intercerebralis. The return to a rhythmic pattern of activity is presumed to occur after the remaining neurosecretory cells regain their normal functioning capacities. In 3 cases, rhythms regenerated several weeks post-operatively (19, 20, and 24 days) which suggests that (in these cases) more drastic damage had been done to the system which required more elaborate and time-consuming repair processes before rhythmicity could be re-established. In this context one might attribute the long period of time required for the reappearance of a rhythm to damage done to the nerve tracts during surgery, and the need for subsequent regeneration of the tracts before a return of the rhythmicity.

At present, it is still unclear whether or not the pars intercerebralis—especially the neurosecretory cells—*directly* controls the locomotory rhythm for the following reasons: (1) Neurosecretory cells in the brain play several important roles in other metabolic functions; such as stimulation of protein synthesis (Thomson and Møller, 1959, 1963; Hill, 1962), triggering of the prothoracic gland hormone, the promotion of water retention and stimulation of oviposition (*cf.* Van der Kloot, 1960). The observed arrhythmicity might, therefore, be a secondary effect. (2) To date, we have examined histologically the brains of only 3 arrhythmic animals. In these preparations, most of the corpora pedunculata has been ablated along with the pars intercerebralis. Since these carry important fiber connections including those from the optic lobes (*cf.* Horridge, 1965) further experiments involving these areas may be required.

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We are grateful to Professor B. Scharrer for her criticism and suggestions.

SUMMARY

1. Ablation of the region of the pars intercerebralis of the cockroach brain induced arrhythmicity of locomotory activity in the animal. Evidence is presented which demonstrates a relationship between the neurosecretory cells of the pars intercerebralis and the circadian rhythm.

a. Surgical removal of the pars intercerebralis, including the lateral and medial neurosecretory cells, results in arrhythmicity and an increased level of activity.

b. In a large number of cases where normal activity and rhythms regenerated post-operatively, neurosecretory cells could be demonstrated histologically to be present and presumably functioning.

2. The suggestion is made that the pars intercerebralis acts as a rhythmic suppressor of general locomotory activity resulting in rhythmic locomotory behavior.

3. In animals where rhythms "regenerate" several weeks post-operatively, the speculation was made that biological regenerative processes, such as regeneration of several nerve tracts of a few remaining neurosecretory cells, in addition to the general recovery from and adjustment to the brain surgery, are the time-consuming processes which must occur before rhythms manifest themselves.

4. Insertion of a gel containing actinomycin D into the pars intercerebralis induced arrhythmicity in the animal. However, when actinomycin D gel was implanted into the fifth abdominal ganglion normal activity and rhythm continued unchanged; when gel containing eosin instead of actinomycin D was inserted into the pars intercerebralis, rhythm regenerated post-operatively.

5. It is still unclear if the corpora pedunculata play some role in activity rhythms, and if secondary effects following ablation of the neurosecretory cells are responsible for inducing arrhythmicity in the animal.

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STUDIES OF THE EFFECT OF IRRADIATION OF CELLULAR PARTICULATES. VI. COMPARISON OF UNCOUPLING AT THE THREE PHOSPHORYLATING SITES[1]

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It is well established that exposure of rats to whole-body irradiation results in the uncoupling of oxidative phosphorylation. Mitochondria isolated from various organs show a decreased P:O ratio, so long as the assay is performed at least eight hours after irradiation (Potter and Bethel, 1952; van Bekkum, 1957; Yost, Glickman and Beck, 1964). The majority of this work has been done by measuring uncoupling at the level of the oxidation of cytochrome-*b* (succinate substrate), while a few workers have worked with the initial step, primarily using glutamate as a substrate to reduce pyridine (Hall, Goldstein and Sonnenblick, 1963; Goldstein and Hall, 1965). Recently, we have demonstrated uncoupling at the terminal step, the oxidation of cytochrome-*c* reduced by ascorbate (Yost, Robson and Yost, 1967). These last results indicated that the terminal step in the phosphorylation chain is the most sensitive to uncoupling by ionizing radiations, and this, in turn, suggested the possibility that all of the observed uncoupling at other sites might be merely a reflection of damage done to the terminal step.

There are a number of possibilities for the inactivation of the phosphorylating mechanism. To cite the most obvious, it is possible that all of the uncoupling occurs in the terminal step of the chain. If this were the case, the highest percentage inactivations would be observed when using ascorbate as a substrate; the inactivation at the second step (succinate substrate) should be approximately one-half that observed with the ascorbate; and the inactivation observed in the primary step (glutamate substrate) would be only one-third that observed with ascorbate. If, on the other hand, a higher percentage inactivation were observed in the initial or second step, such an hypothesis would be untenable. Therefore, it seemed worthwhile to make comparative studies of the uncoupling of all three sites to determine whether the inactivation occurs at that one common to all three pathways.

Unfortunately, we cannot use the results obtained previously by various investigators to make the necessary comparisons. The variations in technique are sufficient to cause a rather wide variation in results. In fact, it is difficult to take results from any one laboratory, obtained at different times, and compare them (Yost, Robson and Yost, 1967). Thus, it seemed wise to observe the uncoupling of all three steps in one laboratory, under a single set of conditions, so that comparative studies of the effect of uncoupling on the three different phosphorylating sites could be made.

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MATERIALS AND METHODS

All experiments were carried out with male, albino rats of the Sprague-Dawley (Gofmoor Farms) and CD (Charles River Sprague-Dawley) strain, weighing between 170 and 210 grams. The animals were housed in large steel cages and fed Purina Lab Chow *ad libitum.*

The rats were exposed to 1000 r of γ-radiation delivered from a 2000 Curie Cs^{137} source, filtered through one-half inch of Lucite. The dose rate was 80 r/min. In general, the rats were irradiated in a small, plastic screened cage containing two rats. Control and experimental animals were given exactly the same treatment with the exceptions of exposure to irradiation.

The procedures used to isolate mitochondria from spleen and liver have been described previously (Yost, Robson and Yost, 1967). The efficiency of oxidative phosphorylation was measured by use of the P:O ratio. For assay of the first step on the phosphorylating chain, glutamate was used as a substrate, and the medium was prepared according to the method of Hunter (1955). For assay of the second step, succinate was used as the substrate. With spleen mitochondria, the medium of Thomson (1964) was used, with the exception that the fluoride concentration was 26 μmoles per vessel. For assays of liver mitochondria, the medium of Yost and Robson (1959) was used with the modification that 36 μmoles phosphate and 65 μmoles of KF were used in each vessel. For assay at the terminal step, ascorbate was used to reduce cytochrome-*c*. Measurements were made using the method of Lehninger, Hassan and Sudduth (1964), with the exception that 26 μmoles of KF and 0.03 μmoles of cytochrome-*c* were used. In assays of the terminal step, it is important that cytochrome-*c* of very high purity be used; throughout, we always used Sigma 100%. Oxygen uptake was measured using a Warburg respirometer at 25° C. Readings were taken until 8–12 μatoms of oxygen had been consumed (usually 30 minutes), after which the reactions were stopped with TCA. Since we are interested only in the relative efficiency of the phosphorylating system, the oxygen consumption was held constant in each run and

TABLE I

A comparison of the uncoupling at each of the three steps in mitochondrial phosphorylation by irradiation

	I Glutamate			II Succinate			III Ascorbate		
	No. runs	Ave. P:O	% Inact.	No. runs	Ave. P:O	% Inact.	No. runs	Ave. P:O	% Inact.
Spleen									
Control	15	3.1	16**	12	2.0	25**	19	0.62	36**
Irradiated		2.6			1.5			0.40	
Liver									
Control	19	3.0	0	15	1.8	11*	25	0.94	16**
Irradiated		3.0			1.6			0.79	

* $P < 0.01$.
** $P < 0.001$.

Table II

The effect of varying swelling time and time of assay on the uncoupling at the terminal step of oxidative phosphorylation

	Time in hypotonic sucrose		
	5 min.	10 min.	15 min.
No. runs	3	3	3
Control	0.96	1.00	1.00
γ	0.63	0.70	0.67
Inactivation	34%	33%	33%

	Post-irradiation time of assay	
	24 hrs.	72 hrs.
No. runs	6	6
Control	0.93	1.05
γ	0.59	0.74
Inactivation	36%	30%

the time on the Warburg was allowed to vary. Phosphate determinations were made by the method of Lowry and Lopez (Glick, 1949). All phosphate determinations were made in duplicate. All assays were conducted 20 to 24 hours post-irradiation.

Results

The data in Table I indicate that, taken individually, all three steps are significantly uncoupled by exposure to ionizing radiation when measured in mitochondria isolated from spleen. However, we were unable to observe any significant uncoupling of the primary step in liver mitochondria, under the conditions used in these experiments. As indicated by our previously reported data, the terminal step seems to be significantly more sensitive than the other two, and the initial step is the least sensitive.

Since Hall, Goldstein and Sonnenblick (1963) have reported the uncoupling of the primary step in liver at times lower than 24 hours post-irradiation, we felt that it would be advisable to determine whether we could observe uncoupling immediately post-irradiation. We chose 3 hours post-irradiation as a time that had given them a significant effect. Six runs were made. The controls averaged 3.1, varying between 2.9 and 3.3; whereas the experimentals averaged 3.2, varying between 3.0 and 3.5. Thus, it would appear that under our conditions, we cannot observe uncoupling in the primary step (in liver). In addition to these experiments, we made four runs assaying the uncoupling at the primary step in spleen at 3 hours post-irradiation. The controls averaged 2.8 (2.7–2.8); whereas the treated average was 2.7 (2.5–2.9). There is no significant difference between the two, and it seems unlikely that any significant uncoupling can be observed, this early, with spleen glutamate. These results are in agreement with those of Thomson, Nance and Bordener (1966).

To obtain adequate P:O ratios at the terminal step, it is necessary to "soak" the extracted mitochondria in hypotonic sucrose to increase the permeability to reduced cytochrome-*c*. We were concerned that this might accentuate the effect of the irradiation by slightly uncoupling the system. Consequently, studies were conducted on the effect of varying the time of treatment with hypotonic sucrose. The data in Table II indicate that the time in hypotonic sucrose does not have any appreciable effect on the P:O ratio, or on the level of inactivation. The normal time of soaking in hypotonic sucrose is 5 minutes (in no case longer than 7 minutes), and thus, whatever variations there are in the data cannot be attributed to the hypotonic treatment. In addition, Table II presents data showing that the effect of the irradiation on the uncoupling of the terminal step is relatively long-lasting as has been previously demonstrated for the uncoupling at the second step (Yost, Glickman and Beck, 1964).

DISCUSSION

The data presented in this paper indicate that the majority of uncoupling of oxidative phosphorylation by ionizing radiation can be attributed to uncoupling at the terminal step. Obviously, it is difficult to assume that any particular value has special validity. Only the relationship of the values is important. Thus, if we arbitrarily pick the terminal step, we can set the expected values of inactivation for the other two steps relative to that empirically-derived value. If all the inactivation were coming from the terminal step, one would expect (in the spleen) that the second step would show one-half of the inactivation observed with the terminal step alone, or 18%. Similarly, one would expect one-third of the inactivation in the primary step, or 12%. The difference between the "expected" 12% and the observed 16%, in the first step, does not seem sufficient to suggest that there is any uncoupling of the primary step itself. At the second step, however, there seems to be more inactivation than would be expected simply from uncoupling of the terminal step alone. This suggests that the second site itself is partially damaged. However, it must be clear that the damage is relatively slight, since the majority of the inactivation observed can be accounted for simply on the basis of the inactivation occurring at the terminal step.

Approximately the same results as those discussed above were obtained with liver mitochondria. One would expect, working from the terminal step backwards, values of 16%, 8% and 6%. Failure to observe any inactivation at the primary step is difficult to explain. However, it may be that with "expected" percentage inactivations that low, even larger numbers of runs than those done for this paper would be necessary to demonstrate inactivation. The relatively good agreement for the second step suggests that any uncoupling of liver mitochondrial phosphorylation by exposure to ionizing radiation resides in the terminal step alone.

Further comment should be made on the disagreement between the results obtained with liver mitochondria and those obtained by Hall, Goldstein and Sonnenblick (1963). As we have pointed out elsewhere (Yost, Robson and Yost, 1967), the values obtained in studies of the uncoupling of phosphorylation are subject to a number of experimental modifications. In fact, the values that we now achieve in this laboratory are much lower than those we initially obtained (for example, compare Yost, Glickman and Beck, 1964). In addition to differences in techniques, it is probable that commercially obtained rats are becoming progressively more resistant to some of the abscopal effects of irradiation, not through any design of the breeders but through improvement in overall disease resistance, etc. The fact that we failed to observe inactivation of liver glutamate should not be taken as an indication that experiments by other workers are invalid. Rather, they should serve to point to the fact that comparisons can be made only within one set of data. Considering the possible sources of variation in P:O ratios, this is hardly a surprising conclusion.

In 1960, we put forward an hypothesis that the effects of irradiation on the uncoupling of oxidative phosphorylation were largely abscopal in nature (Benjamin and Yost, 1960). At that time, it was suggested that it might be to the advantage of an organism to accelerate its metabolism, in order to provide the necessary intermediates required for the restitution of damage. If such a mechanism were operating, one might expect that the terminal step would be the most sensitive, since uncoupling the terminal step achieves the release from "tight coupling" control with the least possibility of damage to the rest of the phosphorylating chain. Thus, the observation that the terminal step is the most sensitive to the effects of whole-body irradiation is consistent with the hypothesis that the observed uncoupling is merely part of a more generalized response to stress.

SUMMARY

White male rats of Sprague-Dawley strain were exposed to 1000 r total-body γ-irradiation. Measurements of the uncoupling at each of the three phosphorylating sites in mitochondria isolated from liver and spleen were made. The results indicate that the terminal step (oxidation of reduced cytochrome-*c*) is the most sensitive of the three and that the great majority, if not all, of the observed uncoupling may result from damage to this step.

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